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**THE ROLE OF SIGNAL TRANSDUCER AND ACTIVATOR OF
TRANSCRIPTION-3 (STAT-3)
IN
ISCHAEMIC AND PHARMACOLOGICAL POSTCONDITIONING**

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DECLARATION

I, Sarin Somers, hereby declare that the work on which this thesis is based is my original work (except where acknowledgement indicates otherwise), and that neither the whole work nor any part of it has been, is being, or is submitted for another degree in this or any other university.

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ABBREVIATIONS

α	alpha
β	beta
δ	delta
γ	gamma
κ	kappa
μ	mu
5-HD	5-hydroxydecanoate
7TM	seven transmembrane domain receptors
ACE	angiotensin converting enzyme
Adeno-PostC	adenosine postconditioning
ADP	adenosine diphosphate
AG	AG490/ tyrphostin
AMI	acute myocardial infarction
ANOVA	analysis of variance
ANT	adenine nucleotide translocator
AR	adenosine receptor
ATP	adenosine triphosphate
AU	arbitrary units
Bad	Bcl-2 associated death agonist
Bcl-2	B-cell lymphoma-2
Bcl-xL	B-cell lymphoma-xL
Bk-PostC	bradykinin postconditioning
COOH	carboxyl terminus
CREB	cAMP Response Element-Binding
CRIF1	CR-6-Interacting Factor
CsA	Cyclosporine A

CuSO ₄ .5H ₂ O	copper sulphate pentahydrate
CVD	Cardiovascular disease
CYP-D	Cyclophilin-D
DADLE	D-Ala 2', D-Leu 5'- enkephalin acetate salt
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
D-PostC	DADLE postconditioning
DTT	Dithiothreitol
dH ₂ O	distilled water
ddH ₂ O	double distilled water
EDTA	Ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ETN	ethanolamine
Fig.	Figure
FOXO-1	Forkhead transcription factor-1
GAPDH	Glyceradehyde-3-phosphate dehydrogenase
GCPR	G coupled protein receptor
GDP	guanine diphosphate
GSK-3β	glycogen synthase kinase -3 beta
GTP	guanine triphosphate
H ⁺	hydrogen ion
HDL	high density lipoprotein
IC	ischaemic control
IgG	Immunoglobulin G
IHD	ischaemic heart disease
IDL	intermediate density lipoprotein

iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
IPostC	ischaemic postconditioning
JAK	Janus kinase
LDL	Low density Lipoprotein
LIF	Leukemia Inhibitory Factor
LPP	Lipid phosphate phosphatase
KH ₂ PO ₄	monopotassium phosphate
KO	knockout
MEK	Mitogen activated protein kinase Extracellular regulated Kinase
MgCl ₂	Magnesium chloride
mitoK _{ATP}	mitochondrial potassium sensitive adenosine triphosphate channel
MLC2V	Myosin Light Chain isoform-2 Ventricle
MnSOD	manganese superoxide
mPTP	mitochondrial permeability transition pore
MRC	Medical Research Council
NaBr	sodium bromide
NAC	N-acetylcysteine
NaCl	sodium chloride
NaVO ₄	sodium orthovanadate
NH ₂	amino terminus
NH ₄	ammonia
NIH	National Institute of Health
p70s6K	p70-ribosomal s6 kinase
PAGE	polyacrylamide gel electrophoresis
PI3-K	phosphatidylinositol 3-kinase

PIAS	Protein Inhibitors of Activated STAT
PKC	Protein Kinase C
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PD	PD 98059
PMSF	Phenyl Methyl Sulfonyl Fluoride
RISK	Reperfusion Injury and Salvage Kinase
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SOCS	Suppressor of Cytokine Signaling
SDS	Sodium Dodecyl Sulfate
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-Phosphate receptor
SAFE	Survivor Activation Factor Enhancement
SiRNA	Silencing ribonucleic acid
SIRT1	silent information regulator-1
SMase	sphingomyelinase
SPK	sphingosine kinase
SPT	8-(p-sulfophenyl) theophylline
STAT-3	Signal Transducer and Activator of Transcription-3
Taq	Thermus aquaticus
TBS-T	Tris Buffered Saline with 0.1% Tween
TNF α	Tumour Necrosis Factor alpha
TNFR	Tumour Necrosis Factor alpha receptor
TTC	Triphenyltetrazolium chloride
VDAC	voltage dependant anion channel
VLDL	Very Low Density Lipoprotein

W	wortmannin
WHO	World Health Organisation
WT	wildtype

ABSTRACT

Aims: Ischaemic postconditioning (IPostC) is a powerful phenomenon that harnesses the body's innate system to protect the myocardium against ischaemia/reperfusion injury. The precise signalling mechanisms that elicit this cardioprotective effect are unclear but suggest a key role for the cell protective Reperfusion Injury Salvage Kinase (RISK) pathway that activates Akt and extracellular signal-regulated kinase-1/2 (Erk1/2). Recently, the powerful prosurvival path named as the Survivor Activation Factor Enhancement (SAFE) pathway, and involving activation of tumour necrosis factor- α (TNF α) and signal transducer and activator of transcription-3 (STAT-3) has been identified in ischaemic preconditioning. In this thesis, we investigated the role of the SAFE pathway in ischaemic and pharmacological postconditioning. Pharmacological postconditioning was achieved with sphingosine-1-phosphate (S1P), a constituent of high density lipoprotein (HDL), or with classic preconditioning pharmacological agents such as bradykinin, adenosine and opioids. Furthermore, we also proposed to investigate a possible interplay between the SAFE and the RISK pathways in S1P mediated cardioprotection against reperfusion injury.

Methods: Isolated mouse hearts from TNF α knockout (TNF $\alpha^{-/-}$) and cardiomyocyte-specific STAT-3 knockout (STAT-3 $^{-/-}$) were subjected to an ischaemia/reperfusion insult and postconditioned with brief periods of ischaemia, bradykinin (100 nM), a specific delta opioid agonist, D-Ala 2', D-Leu 5'-enkephalin acetate salt (DADLE, 100 nM), adenosine (100 μ M), HDL (200 μ g/ml) or sphingosine-1-phosphate (10 nM). Hearts were stained with triphenyltetrazolium chloride and infarct size was analyzed with computerized planimetry. Cytosolic, nuclear and mitochondrial protein levels of STAT-3, Akt, and Erk1/2 were determined by western blot analysis.

Results: Ischaemic postconditioning and all pharmacological postconditioning mimetics reduce infarct size in WT mice but failed to protect STAT-3 $^{-/-}$ and TNF $\alpha^{-/-}$ mice (except for adenosine in TNF $\alpha^{-/-}$). This protective effect was associated with a phosphorylation and therefore activation of nuclear and/or mitochondrial STAT-3. Inhibition of the RISK path in S1P induced cardioprotection resulted in a lack of activation of the SAFE path, and vice versa. Furthermore, the RISK path failed to be activated with S1P in STAT-3 $^{-/-}$ mice.

Conclusion: Our data revealed a critical role for TNF and STAT-3 in ischaemic and pharmacological postconditioning-induced cardioprotection. The dual activation of the SAFE and RISK pathways confirms the existence of multiple prosurvival pathways which may present novel therapeutic strategies to protect against reperfusion injury.

A. INTRODUCTION

1.0 Global Implications for cardiovascular disease

In 1628, William Harvey made the profound discovery of the human blood circulatory system setting in motion an ideology that was further built upon and is still upheld by the medical world today (Garber, 2008). He described the heart as a pump through which blood was continually circulated from the heart to the lungs and back to the heart and then through the body. The concept of cardiovascular disease, at the time was barely fathomable.

However, this changed with the arrival of the 20th century. Due to the increase in industrialization, urbanisation and lifestyle changes, cardiovascular disease became the dominant disease in the world. Today, it accounts for approximately 30 percent of deaths worldwide (Libby, 2008).

Cardiovascular disease (CVD) is a broad term that describes diseases affecting the heart and its circulatory system. Commonly, it refers to those related to atherosclerosis (the hardening of arteries or blood vessels due to the accumulation of fatty deposits). These include coronary heart disease, stroke and inflammatory heart disease. Amongst them, ischaemic heart disease (IHD) is one of the most prevalent and is predicted by the World Health Organisation (WHO) to become the leading global burden by 2030 (Mathers and Loncar, 2006).

Fifty years ago, ischaemic heart disease was considered rare in sub-Saharan Africa. Today, it has emerged as the eighth leading killer in this region. In people over 60 years, IHD is ranked as the leading cause for mortality in men and second in woman (Figure 1) (Mensah, 2008). The increase in high blood pressure, diabetes mellitus, tobacco use, obesity and dyslipidemia are contributing factors of atherosclerosis which have impacted on the escalating rate of IHD (Reddy and Yusuf, 1998). Furthermore, a study conducted by the Medical Research Council (MRC) listed IHD as the primary non-communicable burden of disease in South Africa (Bradshaw et al., 2003).

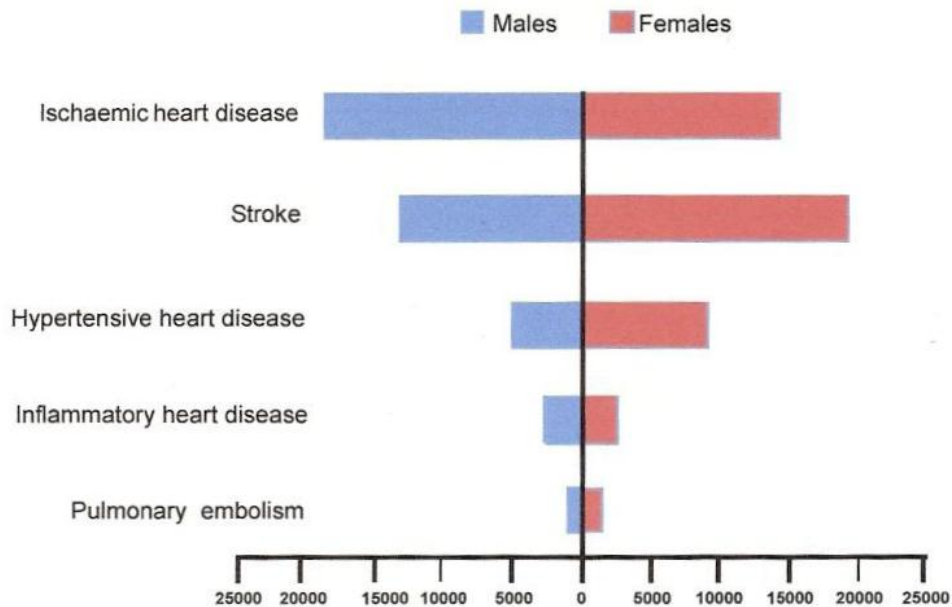


Fig 1. Ischaemic heart disease has been ranked as the leading cause of cardiovascular disease mortality by the South African Medical Research Council. Adapted from (Norman et al., 2007).

1.1 Ischaemic heart disease

Ischaemic heart disease is characterized as the reduced supply of oxygenated blood to the myocardium (heart muscle) and is caused by the partial or complete obstruction of a coronary artery to the heart (Opie, 2004). If this coronary occlusion is allowed to persist, the resultant dead cells form an infarct that may proceed to total necrosis (Figure 2). Additional pathology includes ischaemia-induced contractile dysfunction and hypertrophy. Therefore, it is vital that a timely intervention be implemented to restore the blood flow and rescue the ischaemic myocardium from an acute myocardial infarction.

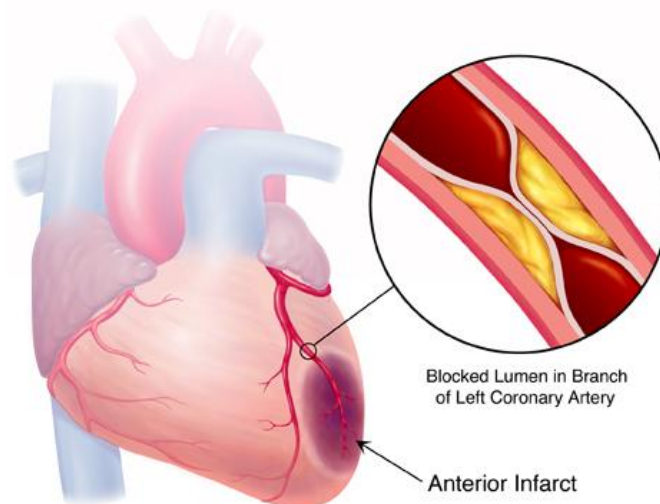


Fig 2. Coronary artery occlusion leads to development of a myocardial infarction. Obstruction of oxygenated blood flow causes myocardial necrosis and eventually ischaemic heart disease. (<http://nursingcrib.com/nursing-care-plan/nursing-care-plan-myocardial-infarction/>)

2.0 Reperfusion Injury

Restoration of coronary blood flow following ischaemic injury may be accomplished by myocardial reperfusion. This procedure is achieved via thrombolytic therapy (use of pharmacological agents to break the blood clot) or primary percutaneous coronary intervention (PCI, insertion of a stent to open the affected artery via balloon angioplasty). Paradoxically, these effective strategies used to reduce the myocardial infarct size, cause reperfusion injury and reduce the beneficial effects of reperfusion.

Lethal reperfusion injury causes the functional impairment of myocardial contractility (stunning), arrhythmia and death of cardiomyocytes which further increases infarct size (Opie, 1989, Opie, 2004). The additional release of reactive oxygen species (ROS) and calcium overloading upon reperfusion is also detrimental to the myocardium (Zweier, 1988, Kloner, 1993, Piper et al., 1998).

Three potential initial causes of lethal reperfusion injury exist, aside from ROS. They are, as follows (Piper et al., 1998):

- 1) *The re-energization of the ischaemic cell.* Upon reperfusion, oxygen is resupplied to the mitochondria which lead to the hypercontracture of cardiomyocytes elicited by high levels of calcium in the cytosol.

Mitochondria are responsible for the production of ATP which is synthesised by means of the oxidative phosphorylation. During ischaemia, calcium and sodium accumulate within the cytosol of myocardial cells. Reoxygenation as a result of reperfusion reactivates the calcium pump of the sarcoplasmic reticulum and the sodium pump of the sarcolemma which function to restore the homeostasis of these ions within the mitochondria and cytosol. However, if the pumps are damaged during the ischaemic event, the imbalance would lead to intracellular calcium overload and ATP production would cease. In addition, hypercontracture of the sarcolemma could lead to membrane rupture and cell death.

2) *The rapid normalization of tissue pH.* The intercellular and interstitial space acidosis that is caused by the anaerobic metabolism during the prolonged ischaemia is disrupted by reperfusion. In an attempt to restore pH balances, calcium overload may occur and cause increased hypercontracture and cell injury.

The cytosolic and intracellular pH is lowered to acidic levels during ischaemia as a consequence of anaerobic metabolism and breakdown of ATP. Reperfusion leads to the activation of the sodium/ hydrogen exchanger and bicarbonate/sodium symporter which function to reduce the acidity. During this pH restoration, the sodium/ calcium pump may be activated and the attempt to remove excess sodium from the cell may cause an intracellular calcium overload.

3) *The rapid normalization of tissue osmolality.* During ischaemia, there is an accumulation of end-products from anaerobic metabolism in the intracellular and extracellular space. The extracellular osmolality is rapidly normalized by reperfusion and thus a transsarcolemmal osmotic gradient is created. This leads to cell swelling and fragility which favour rupture of the sarcolemma.

These events may be further propagated by cell-to-cell interactions and eventually progress to necrosis. A high concentration of reactive oxygen species can also cause lead to greater infarct size by causing membrane lipid and DNA oxidation (Zweier et al., 1987). Experimental studies with Heptanol, a gap junction uncoupler during the first minutes of reperfusion, limited the spread of necrosis and significantly reduced infarct size in the in situ pig heart following a coronary occlusion (Garcia-

Dorado et al., 1997). It was important to find the correct intervention that reduced and did not simply delay the manifestation of irreversible injury.

3.0. The Concept of Conditioning

3.1. Definition: Ischaemic pre- and postconditioning

Over the past few decades, considerable attention has been focused on the development of novel strategies to attenuate myocardial ischaemia-reperfusion injury and improve the outcome post-MI. Acute myocardial infarction (AMI) by prolonged ischaemia resulted in severe necrosis whereby dead cells were unable to be replenished by division of surviving cardiomyocytes. The unsatisfactory benefit of reperfusion therapy contributed more than alleviated the myocardial infarct size.

In 1986, Murry et al. published a seminal paper in which they described a phenomenon whereby four cycles of five minutes of ischaemia with intermittent reperfusion prior to a subsequent and prolonged ischaemia attenuated infarct size by 75% in the canine myocardium. Initially, they found that brief periods of ischaemia reduced the rate of adenosine triphosphate (ATP) depletion during subsequent ischaemic episodes (Reimer et al., 1986). Intermittent reperfusion also served to prevent the cumulative effects of ischaemic injury by washing out potentially harmful catabolites such as lactate, hydrogen ions (H^+) and ammonia (NH_4). The reduction in ATP depletion was associated with the limitation in infarct size. Given these findings and that the procedure could be reproduced successfully, this cardioprotective phenomenon was termed 'ischaemic preconditioning' (IPC) (Murry et al., 1986).

This model of cardioprotection is referred to as 'classic' or early preconditioning and is initially transient, with preconditioned state lasting for only 1-2 hours (Figure 3) (Murry et al., 1991). However, within 24 hours of the preconditioning stimulus, a late phase of protection, known as delayed preconditioning, is evident but is less robust and more prolonged, lasting up to 72 hours after the preconditioning stimulus (Baxter et al., 1997).

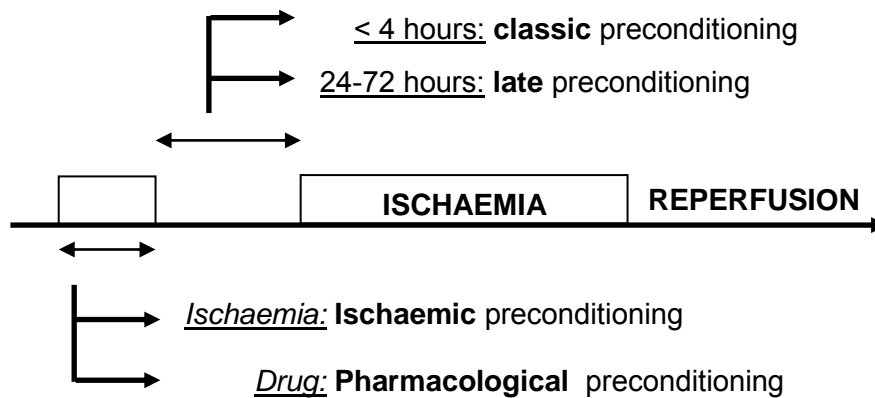


Fig 3. Schematic representation of classic and delayed preconditioning

Since its inception, researchers have experimented with ischaemic preconditioning in different animal models and conditions to elucidate a possible signalling mechanism. The discovery of a signal transduction pathway would allow for the development of novel drug therapeutics. The protective benefits of IPC have also been successfully reproduced with the introduction of a pharmacological agent stimulus prior to the ischaemic event. Adenosine, bradykinin and opioids were among the early agonists that mimicked IPC and are discussed later in this section.

Ischaemic preconditioning demonstrated that brief episodes of ischaemia could protect the myocardium from a subsequent lethal ischaemic event (Murry et al., 1986). Although researchers have been consumed with delineating its signalling mechanisms and discovering pharmacological mimetics in the past decade, it is impossible to predict the index ischaemic period. This unpredictability makes preconditioning a difficult strategy to implement in a clinical setting against myocardial ischaemia/ reperfusion injury. However, targeting the reperfusion phase is an appealing option. In 2003, Vinten-Johansson's group elucidated that subjecting the *in vivo* canine myocardium to 3 x 30 second ischaemic episodes with intermittent reperfusion, dramatically reduced myocardial infarct size (Zhao et al., 2003). The observed levels of cardioprotection induced by this phenomenon termed as ischaemic postconditioning (IPostC) were strongly comparable to those in preconditioning (Figure 4).

The protective benefits of IPostc have also been established in other animals such as rats, mice, rabbits and pigs (Kaljusto et al., 2006, Schwartz and Lagranha, 2006,

Skyschally et al., 2009, Yang et al., 2004). Interestingly, infarct size reductions by IPostC in large animals such as dogs (50-80%) are greater than in smaller animals (mice, rats and rabbits (~30%). This is more variable than the 50-80% infarct sparing effect in preconditioning (see review, (Vinten-Johansen et al., 2007)). Halkos et al. found that IPC and IPostC offered similar levels of protection in the canine model but not in the rat model where IPC yielded a greater protection (Halkos et al., 2004). It is possible that a species discrepancy may exist which may be due to metabolic or genetic differences and that an adjustment in the IPostC algorithm may be required. Nevertheless, IPostC displays a promising clinical strategy in the attenuation of acute myocardial infarction.

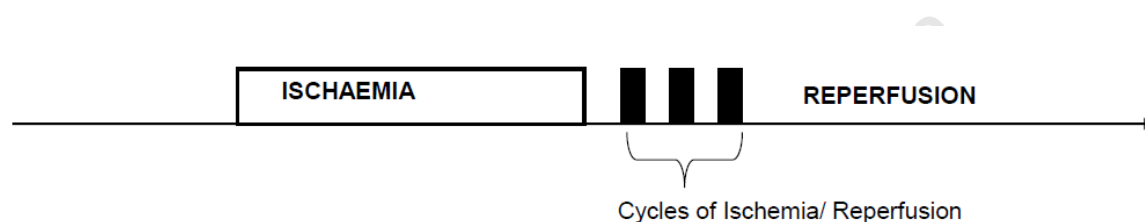


Fig 4. Schematic representation of a postconditioning protocol

3.2 Clinical relevance

Percutaneous coronary intervention (PCI) and thrombolytic agents are used to restore reperfusion and reduce infarct size in patients presenting with an acute myocardial infarction. However, lethal reperfusion injury occurs, leading to structural and functional damage to the myocardium, as discussed earlier. Ischaemic preconditioning has been demonstrated to be highly effective in reducing infarct size and improving functional recovery in the heart. The unpredictable nature of prolonged ischaemia limits the application of preconditioning in clinical practice. Preconditioning may be implemented in surgery where it is expected to be a long ischaemia such as during cardiac bypass or transplantation surgery. No foreknowledge is required with postconditioning. In 2005, Staat et al conducted a clinical trial that examined the prospect of postconditioning in patients with AMI who were randomized to receive four 1 minute cycles of inflation-deflation with an angioplasty balloon following stent placement. Infarct size was successfully reduced (Staat et al., 2005).

Although small clinical proof-of-concept studies suggest that ischemic postconditioning can protect the human heart against lethal reperfusion injuries, larger clinical trials testing ischemic or pharmacological postconditioning are needed to test the clinical outcome of this phenomenon.

3.3. Mechanisms of Conditioning

3.3.1 G-Protein coupled receptors

G-protein coupled receptors (GPCRs), also known as the seven-transmembrane domain receptors (7TM) or serpentine receptors, are a diverse and abundant type of cell surface receptors found in eukaryotes (Hall et al., 1999). Structurally, they consist of seven alpha-helical protein domains that span the cell membrane, with an extracellular amino terminus and a cytosolic carboxyl terminus (Figure 5) (Schertler et al., 1993, Palczewski et al., 2000).

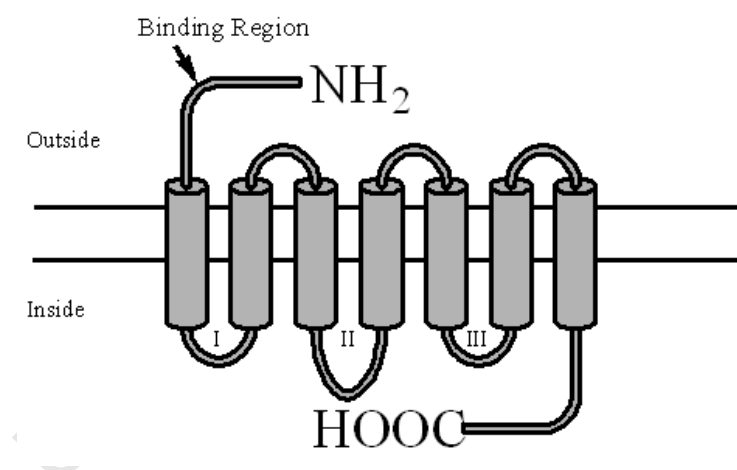


Fig 5. Structure of a typical G coupled protein receptor. GPCRs contain seven transmembrane domains, with an extracellular amino terminus and an intracellular carboxyl terminus. (<http://images-mediawiki-sites.thefullwiki.org/03/9/1/2/441015484151498.gif>) *NH₂*, amino terminus; *COOH*, carboxyl terminus

GPCRs are receptors for G proteins which belong to a superfamily of proteins that bind guanine triphosphate (GTP), guanine diphosphate (GDP) or guanine nucleotides. Upon ligand binding, the GPCR undergo a conformational change which is transmitted throughout the helices to the cytosolic side of the membrane (Figure 6), (Ballesteros et al., 2001, Shapiro et al., 2002). The activated receptors switch on

G proteins by catalyzing the exchange of GTP for GDP. GPCRs are deactivated by phosphorylation of serine or threonine residues in their C-terminus (Libby, 2008, Hermans, 2003). The active trimeric GTP-bound G protein disassociates into G_{α} -GTP and $G_{\beta,\gamma}$ -GTP subunits. The G_{α} -subunit switches on the target before it is hydrolyzed to return the G protein to its inactive form i.e as GDP-bound G protein.

The stimulatory G_s protein and inhibitory G_i protein are both activated via this mechanism but exert opposite effects on the same system. For example, The alpha unit of G_s , in combination with GTP enhance the activity of adenylyl cyclase (responsible for production of second messenger, cyclic adenosine monophosphate (cAMP) that is indirectly required for contractile activity) which is inhibited by the G_i (Ross and Gilman, 1980). G-protein coupled receptors play an important role in the signal transduction processes concerned with mediating cardioprotection.

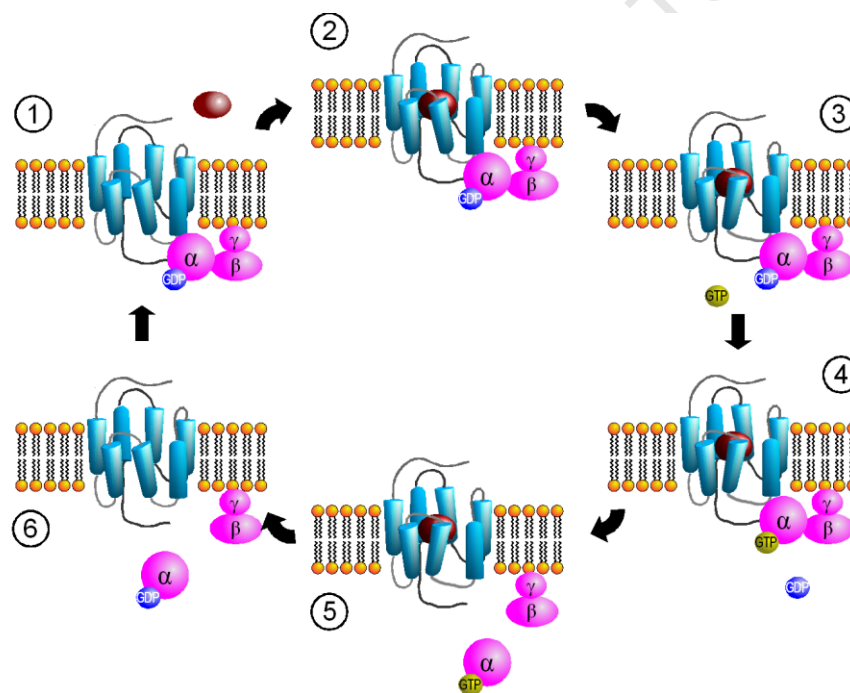


Fig 6. The GDP/GTP- bound G protein cycle (http://en.wikipedia.org/wiki/G_protein)

GPCRs involvement in postconditioning require that 1) the receptors must reside in the target tissue (in this case, the myocardium); 2) the ligand is endogenously produced and increased either during ischaemia or reperfusion; 3) the ligand-receptor interactions may be inhibited either pharmacologically or via genetic knockout or silencing ribonucleic acid (SiRNA) models; and 4) exogenous addition of the ligand must induce protection comparable to IPC or IPostC (see review, (Vinten-

Johansen et al., 2007)). Adenosine, bradykinin and opioids receptors are discussed later in this thesis with regard to postconditioning.

3.3.2. The RISK pathway

The Reperfusion Injury Salvage Kinase (RISK) pathway consists of protein kinases, phosphatidylinositol 3-kinase (PI3-K), Akt, extracellular regulated kinases (ERK) and glycogen synthase kinase-3 β (GSK-3 β) (Hausenloy and Yellon, 2004, Yellon and Baxter, 1999). The involvement of these kinases was postulated based on their prosurvival properties against reperfusion injury (Yellon and Baxter, 1999).

PI3-K/Akt was first shown to be activated by phosphorylation following an ischaemic preconditioning stimulus in the isolated rat heart (Tong et al., 2000). Inhibition of PI3K with Wortmannin or LY 294002 abolished IPC induced protection. Although ERK1/2 was also phosphorylated in response to an IPC stimulus before the prolonged ischaemic event (Fryer et al., 2001), we and others have shown that inhibition of ERK1/2 did not influence the preconditioning outcome (Somers, 2009).

These kinases have also been investigated as potential mediators of IPC at the time of reperfusion in a rat heart model (Hausenloy et al., 2005a). This infarct limiting study involved the inhibition of PI3K/Akt and ERK with LY 294002 and PD 98059, respectively. Activation of these prosurvival kinases prior to the index ischaemia and at the time of reperfusion could imply a biphasic response to IPC. It has been suggested that the early activation during the IPC stimulus would serve as a primer for the kinase phosphorylation observed at the time of reperfusion (Hausenloy et al., 2005b). Alternatively, an intermediary such as adenosine or ROS may exist to facilitate this prosurvival mechanism.

Inhibition of PI3-K at reperfusion with wortmannin or LY 294002 abrogated the postconditioning effect in the isolated perfused rat heart (Tsang et al., 2004). The involvement of the PI3-K/Akt pathway was confirmed by Yang et al. in the isolated perfused rabbit heart model (Yang et al., 2005). This group also reported a loss of IPostC induced protection with the inhibition of the MEK/Erk 1/2 pathway by the MEK inhibitor PD 98059 (Yang et al., 2004). In contrast, Darling et al. reported that there was no attenuation in infarct size in the isolated perfused rabbit model with PI-3K

inhibition by LY 294002 but PD 98059 abrogated the infarct sparing effect of IPostC (Darling et al., 2005).

Glycogen synthase kinase 3 belongs to a family of conserved serine/threonine kinases present in eukaryotes. It exists in two isoforms in humans, i.e GSK-3 α (51 kDa) and GSK-3 β (47 kDa) (Grimes and Jope, 2001, Harwood, 2001). Although both isoforms are expressed in the heart, GSK-3 β is the most extensively studied (Henry and Killilea, 1994). GSK-3 β was originally described for its ability to inhibit glycogen synthesis (Embi et al., 1980) but has since been discovered to be involved in a host of cell functions, including metabolism, gene expression and cell integrity (Grimes and Jope, 2001).

GSK-3 β is constitutively active in the cytosol of cardiomyocytes. Upon phosphorylation at its serine 9 residue, GSK-3 β is rendered inactive. This phosphorylation may be achieved by multiple pro-survival signalling kinases such as Akt, Protein Kinase C (PKC), or ERK, in the reduction of ischaemia reperfusion injury (Murphy and Steenbergen, 2007, Sugden et al., 2008, Tong et al., 2002). Additionally, the inactivation of GSK-3 β also inhibits the opening of the mitochondrial permeability pore (discussed later in this chapter). Murphy et al. established that phosphorylation of the serine 9 residue by ischaemic preconditioning led to inhibition of GSK-3 β (Tong et al., 2002). Additionally, the isoform non-selective pharmacological inhibition of GSK-3 also protected against ischaemia reperfusion injury and successfully reduced infarct size.

Downstream prosurvival factors of the RISK pathway such as p70S6 kinase, PKC ϵ , endothelial nitric oxide synthase (eNOS) and GSK-3 β are potential mediators of IPostC induced protection (Hausenloy and Yellon, 2006, Burley and Baxter, 2009, Gomez et al., 2008). It is likely that these downstream targets of the RISK pathway may also be involved in the inhibition of apoptosis rather than directly by the RISK pathway, itself.

3.3.3. The role of mitochondria in conditioning

Mitochondria are prominently known as the energy powerhouses of the cell. They are responsible for the production of adenosine triphosphate (ATP) by respiration

(Citric Acid cycle) which is used to drive metabolic processes, including regulation of membrane potential; apoptosis programmed cell death and calcium signalling. The cardioprotective properties of the mitochondria have piqued the interests of many researchers who believe the organelle to be the final deciding factor or end effector in mediating a successful recovery from ischaemic damage. This benefit would be attributed to the opening of the mitochondrial adenosine triphosphate dependant potassium channel (mitoK_{ATP}) and closure of the permeability transition pore (mPTP).

3.3.3.1 Mitochondrial adenosine triphosphate dependent potassium channel

Activation of the mitoK_{ATP} channel plays an important role in preconditioning. It was first discovered by Gross et al. who demonstrated glibenclamide, a K_{ATP} antagonist blocked the protective effects of preconditioning in dog hearts (Gross and Auchampach, 1992). At the time, it was unknown that K_{ATP} channels existed in both the sarcolemma and mitochondria. This was later clarified with use of different models, attributing the cardioprotective role to the mitoK_{ATP} channel (Gross and Fryer, 1999, Garlid et al., 1997, Liu et al., 1998).

ATP is synthesized from ADP in the mitochondria by the re-entry of H⁺ via the F1 apparatus along a strong electrochemical gradient. The opening of the mitoK_{ATP} allows for the inflow of potassium into the mitochondria. The intracellular potassium is exchanged for extracellular H⁺ by the potassium/hydrogen exchanger located on the inner mitochondrial membrane. Hence, the H⁺ enters the mitochondria by means of skipping the F1 apparatus and ATP production is reduced (Oldenburg et al., 2002a). The structure of the mitoK_{ATP} has never been identified.

Interestingly, the opening of the mitoK_{ATP} channel has been linked to the release of reactive oxygen species. Diazoxide, a mitoK_{ATP} channel activator, induced a preconditioning benefit which was abolished with the addition of a free radical scavenger, N-acetylcysteine (NAC) (Forbes et al., 2001). Inhibition of PI-3 Kinase with wortmannin did not block the increased fluorescence of diazoxide (ROS measured with MitotrackerTM probe), suggesting that mitoK_{ATP} channel would be downstream of the RISK pathway (Oldenburg et al., 2002b). Protein Kinase C (PKC)

is also closely involved in preconditioning. When PKC was downregulated with phorbol 12-myristate 13-acetate, diazoxide failed to activate the mitoK_{ATP} channels and precondition the rat heart (Wang et al., 2001b). This would suggestively rule out mitoK_{ATP} as the end effector for preconditioning mediated cardioprotection since PKC signalling would be downstream of mitoK_{ATP} channels.

Blockade of the mitoK_{ATP} by glibenclamide or 5-hydroxydecanoate (5-HD) abolished the postconditioning effect in rabbit and pig hearts (Iliodromitis et al., 2006, Yang et al., 2004, Yang et al., 2005). Pharmacological postconditioning with isoflurane, a volatile halogenated anesthetic, limited infarct size (Chiari et al., 2005, Feng et al., 2005, Krolkowski et al., 2005). This beneficial effect involved the activation of the PI3-K/Akt pathway, GSK-3 β inactivation and inhibition of the mPTP opening. Inhibition of the mitoK_{ATP} channel abrogated the infarct sparing effect.

3.3.3.2 Mitochondrial permeability transition pore

The mitochondrial permeability transition pore (mPTP) is a non-specific channel of the inner mitochondrial membrane. The opening of this channel via a stressful event such as ischaemia-reperfusion injury would mediate cell death as a result of uncoupling oxidative phosphorylation and induced mitochondrial swelling (Griffiths and Halestrap, 1995, Hausenloy et al., 2003, Halestrap et al., 2004). Controversy surrounds the structure of the mPTP. Its core components were originally perceived to consist of voltage- dependant anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin D (CYP-D). However, knockout studies seem to suggest the exclusion of VDAC and ANT (Baines et al., 2007, Kokoszka et al., 2004). CYP-D is still considered an important regulatory component of the mPTP as CYP-D deficient mice subjected to calcium or oxidative stress failed to induce opening of the pore and smaller infarcts were recorded in response to ischaemia-reperfusion injury (Basso et al., 2005, Baines et al., 2005, Nakagawa et al., 2005).

A cardioprotective strategy against reperfusion injury that harnessed this information involved the transient opening and closure of the mPTP. The infusion of a mPTP inhibitor, cyclosporine-A (CsA), at reperfusion protected the isolated rat heart (Hausenloy et al., 2002). Further studies have implicated the mPTP as a prospective

end effector of ischaemic preconditioning (Hausenloy et al., 2004, Javadov et al., 2003). The precise signalling mechanism by which IPC inhibits the mPTP opening remains to be determined although it is likely that protein kinases such as Akt (Bopassa et al., 2006) or GSK-3 β are involved (Juhaszova et al., 2004).

In 2005, Argaud et al. demonstrated the IPostC induced inhibition of the mPTP in isolated rabbit heart mitochondria (Argaud et al., 2005). The PI3-K/Akt pathway was implicated as an important IPostC mediator of mPTP inhibition when inhibition of the prosurvival pathway with wortmannin or LY 294002 prevented the inhibitory effect of IPostC on the mPTP opening (Bopassa et al., 2006). Cyclophilin-D (CYP-D) is a matrix protein that binds to the inner mitochondrial membrane in a calcium and inorganic phosphate regulated process, to facilitate the opening of the mPTP (Di Lisa and Bernardi, 2009). Postconditioning by inhibition of the mPTP with cyclosporine-A (CsA) or IPostC in CYP-D^{-/-} mice, successfully reduced infarct size (Lim et al., 2007).

Inhibition of the mPTP represents a promising therapeutic target for cardioprotection. Piot et al conducted a pilot trial to investigate the potential attenuation in infarct size in AMI patients following CsA treatment (Piot et al., 2008). CsA was administered as an intravenous bolus immediately before coronary artery reperfusion by PCI. Infarct size was assessed by the release of creatine kinase and troponin I during the first three days of reperfusion and by MRI at day 5 after infarction. Based on the results, the authors concluded that CsA administration at reperfusion successfully reduced infarct size and a larger study was suggested to confirm the findings.

4.0. Pharmacological conditioning agents

Translating ischemic pre- and postconditioning from bench to bedside can be difficult. Patients exhibiting different co-morbidities may negate the protective effects. Therefore, pharmacological conditioning presents the advantage of being applied in AMI patients with or without PCI.

4.1. Adenosine

Adenosine is a purine nucleoside and occurs naturally in the intra- and extracellular space, produced either by 5'-nucleotidase which dephosphorylates AMP or by the hydrolysis of S-adenosyl-homocysteine (Fredholm et al., 2001). Adenosine levels

have been shown to be elevated during ischaemia and early reperfusion (Schrader et al., 1977, Miller et al., 1979). It exerts its effects via the activation of its G-coupled protein receptors: A₁, A_{2A}, A_{2B} and A₃ adenosine receptor (AR) subtypes, located in the myocardium and vasculature (Cohen and Downey, 2008). These receptors mediate various responses such as modulation, coronary flow, heart rate, contraction and cardioprotection. Olafsson et al. reported that an infusion of adenosine during reperfusion limited infarct size and improved ventricular function in the canine myocardium (Olafsson et al., 1987). Preconditioning with adenosine successfully reduced infarct size (Liu et al., 1991) and subsequent investigations confirmed protection with pre-ischaemic A₁ and A₃AR agonism (Liu and Downey, 1992, Lasley and Mentzer, 1992, Thornton et al., 1992, Strickler et al., 1996, Carr et al., 1997, De Jonge et al., 2002, Liu et al., 1994, Tracey et al., 1997, Liang and Jacobson, 1998, Germack et al., 2004) in different models and species.

The infarct-sparing effect of postconditioning was abolished in the isolated rabbit heart model when non-selective A₁ receptor inhibitor, PI3-kinase blocker, wortmannin or an antagonist for guanyl cyclase was applied for 20 min starting 5 min before reperfusion (Yang et al., 2005). Kin et al showed that postconditioning the isolate mouse heart, extended the retention time of intravascular adenosine and improved contractile function (Kin et al., 2005). Furthermore, this group used specific antagonists to demonstrate the required activation of A₂ and A₃ receptors but not A₁ receptor mediating a cardioprotective effect following postconditioning in the open chest rat heart model (Kin et al., 2005). Cohen and colleagues used a non-selective A_{2b} receptor agonist to mimic the postconditioning effect in isolated mouse hearts that was inhibited by PKC inhibitor, chelerythrine (Philipp et al., 2006).

4.2. Bradykinin

Bradykinin is a peptide mediator with vasodilatory properties and synthesized from by kinin-kallikrein plasma protein system. Clinically, angiotensin-converting enzyme (ACE) inhibitors are administered to hypertensive patients to increase plasma levels of bradykinin which serves to reduce high blood pressure. There are 2 bradykinin receptor subtypes, namely B₁ and B₂ found in cardiomyocytes. B₂ is constitutively expressed while B₁ is upregulated by hypoxic stress and inflammatory conditions (Baxter and Ebrahim, 2002).

Exogenous administration of bradykinin was shown to induce protection comparable to IPC in the anaesthetized open-chest rabbit preparation with coronary occlusion (Wall et al., 1994). Inhibition of the B₂ receptor with icatibant abolished this protective effect. Bradykinin's ability to mimic IPC has also been successfully reproduced in other animal studies (Feng et al., 2000, Goto et al., 1995, Bugge and Ytrehus, 1996, Starkopf et al., 1997, Feng and Rosenkranz, 1999).

4.3. Opioids

Opium is a poppy seed extract that is derived from the plant, *Papaver somniferum* and contains alkaloid compounds such as morphine that is used therapeutically for its analgesic and pain alleviation properties. The term 'opioids' refer to drugs whose actions are similar to morphine (but may be chemically different) and includes morphine receptor ant- and agonists that act on the opioid system. In the myocardium, there are opioid peptides including met-enkephalins, leu-enkephalins and dynorphins and three major opioid receptors (μ -, δ -, and κ - subtypes).

Similar to adenosine, opioid levels were also found to be increased after ischaemia (Oldroyd et al., 1992, Paradis et al., 1992, Falcone et al., 1993, Maslov and Lishmanov, 1995). Schultz and colleagues administered morphine (non-selective agonist) as an IPC mimetic and were able to confer protection in the *in vivo* rat myocardium (Schultz et al., 1995). This effect was removed in the presence of glibenclamide, implicating the involvement of the mitoK_{ATP} channel. Agonists for δ - and κ -, but not μ -opioid receptors have also been able to induce protection (Schultz et al., 1997, Schultz et al., 1996, Wang et al., 2001a). This is probably due to the abundance of δ - and κ -opioid receptors found in the ventricles of several species (Ventura et al., 1989, Xia et al., 1996). The μ -opioid receptor has, however, been detected in the atrial trabeculae (Bell et al., 2000).

4.4. Sphingosine-1-phosphate

Sphingolipids are an important source for signalling molecules (Merrill et al., 1997). They are bioactive metabolites that are involved in many cellular processes such as cell differentiation, apoptosis and proliferation (Baumruker and Prieschl, 2002,

Spiegel, 1999, Spiegel and Milstien, 2002, Van Brocklyn et al., 1998). Sphingomyelin is a major membrane sphingolipid that is hydrolyzed by sphingomyelinases to ceramide (Figure 7). Ceramide is then metabolized by ceramidases to sphingosine (Baumruker and Prieschl, 2002). Both ceramide and sphingosine are implicated in apoptosis. However, sphingosine can be phosphorylated by sphingosine kinases (SPK) to form sphingosine-1-phosphate (S1P) (Merrill et al., 1997, Hannun, 1994, Kee et al., 2005).

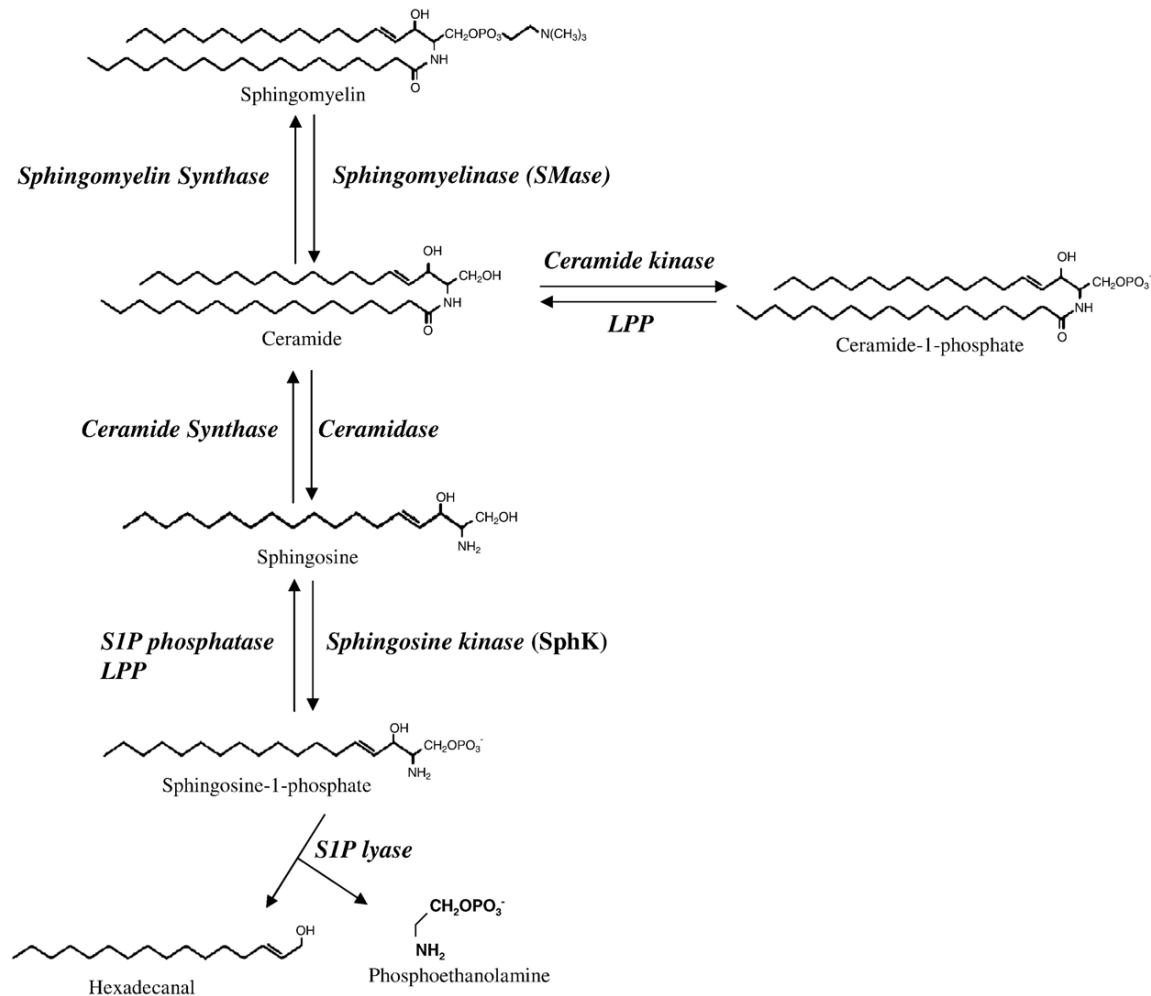


Fig 7. The Sphingolipid metabolic pathway. Sphingomyelin is broken down by hydrolysis into ceramide. Ceramide may be phosphorylated to ceramide-1-phosphate or alternatively converted to sphingosine by ceramidase. Phosphorylation of sphingosine by sphingosine kinase generates sphingosine-1-phosphate. All these reactions can be reversibly metabolized except for the breakdown of sphingosine 1-phosphate to hexadecanal and phosphoethanolamine by S1P lyase. *LPP*, lipid phosphate phosphatase; *S1P*, sphingosine-1-phosphate. (see review, (Melendez, 2008))

S1P is a ligand for specific G-coupled protein receptors. There are five known receptors (S1PR₁₋₅) which have been identified. In the heart, S1PR₁₋₃ activates

signalling pathways that promote cell survival (Zhang et al., 2007a, Means et al., 2008, Mazurais et al., 2002, Alewijnse et al., 2004). S1P treated adult mouse ventricular cardiomyocytes were protected from hypoxia (Zhang et al., 2007a). A S1PR₁ specific antibody was used to implicate the critical involvement of this receptor (Graeler et al., 2003). Pharmacological inhibition studies suggested that S1PR₁ and S1PR₃ were capable of mediating this protective response (Zhang et al., 2007a). The PI3-K/Akt pathway and GSK-3 β were also shown to be involved following inhibition with wortmannin or inactivation by phosphorylation with S1P, respectively. Exogenous S1P protected the intact heart against global ischaemia reperfusion injury by improving cardiac function recovery (Lecour et al., 2002). Ischaemic preconditioning required the activation of SPK1 to reduce infarct size (Jin et al., 2007) which was lost in PKC ϵ ^{-/-} mice (Jin et al., 2004). Preconditioning with ganglioside GM-1 (known activator of the prosurvival kinase PKC ϵ) stimulated the activity of sphingosine kinase to produce S1P (Jin et al., 2002). This study proved that intracellular S1P was dependant on PKC ϵ to induce protection (PKC ϵ ^{-/-} mice were used) compared to exogenous S1P which was cardioprotective independently of PKC ϵ .

In our laboratory we have shown that S1P preconditioning is abrogated in the cardiac specific STAT-3 knockout mice (see Figure 8, unpublished results that I have performed during my PhD). Lecour et al. have previously associated TNF α induced preconditioning with sphingolipid signalling (Lecour et al., 2002). This would suggest the involvement of the protective SAFE pathway in S1P induced preconditioning

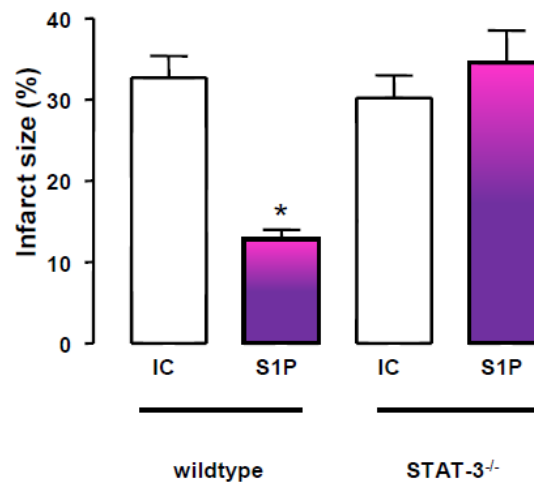


Fig 8. S1P induced preconditioning is dependent on STAT-3. Wildtype hearts subjected to 30min of ischaemia and 45min reperfusion exhibited an infarct size of $32.4 \pm 2.3\%$. However, preconditioning with S1P (10 nM) significantly attenuated infarct size ($12.8 \pm 1.2\%$, $p < 0.05$ vs wildtype control). This effect was abolished in cardiac specific STAT-3 deficient mice ($30.2 \pm 2.8\%$ for control vs $34.5 \pm 4.0\%$ for S1P, $p = \text{ns}$). IC, ischaemic control; S1P, sphingosine 1-phosphate; STAT-3, Signal Transducer and Activator of Transcription-3.

High density lipoproteins (HDL) are a class of lipoproteins that carry cholesterol from the body's tissues to the liver. Elevating its levels with statins, fibrates and other lipid-lowering drugs are used to treat patients with coronary artery disease (Brown et al., 2006, Otvos et al., 2006, 1984). Recently, S1P has been identified as a constituent of HDL and suggested to be responsible for mediating some of its protective effects (Theilmeier et al., 2006, Kimura et al., 2001, Okajima, 2002, Frias et al., 2009, Frias et al., 2010). Levkau and colleagues demonstrated that HDL and S1P protected the *in vivo* mouse myocardium from ischaemia/ reperfusion injury via a nitric oxide and S1PR₃ dependant pathway (Theilmeier et al., 2006). Frias et al showed native and reconstituted HDL with a varied composition of S1P protected cardiomyocytes against doxorubicin induced apoptosis via S1PR₂ but not S1PR₁ and S1PR₃ (Frias et al., 2010).

4.5. Ethanolamine

Ethanolamine (Etn) is a biogenic amine that is stored as membrane lipid, phosphatidylethanolamine. It may be formed by the metabolism of the sphingolipid pathway or endogenous cannabinoid, anandamide. Since cardioprotective molecules, sphingosine 1-phosphate and anandamide are precursors to

ethanolamine, we explored its preconditioning potential in our laboratory (Kelly et al., 2010). Pre-treatment with Etn successfully reduced infarct size in the *in vivo* rat and isolated wildtype mouse heart model. This infarct limiting effect was abolished in cardiac specific STAT-3 knockout mice (Figure 9, results that I have obtained during my PhD). This was the first study, to our knowledge, to suggest a novel cardioprotective role for Etn against ischaemia/ reperfusion injury.

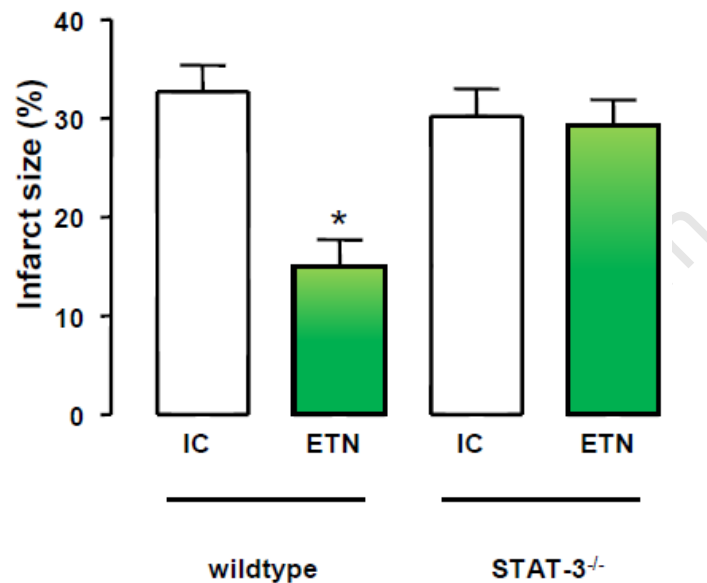


Fig 9. Preconditioning with ethanolamine is abrogated in cardiac specific STAT-3^{-/-} mice. Pretreatment with ethanolamine (0.3 mM) significantly reduced infarct size compared to the ischaemic control in wildtype mice (32.7±2.7% for control vs 15.0±2.7% for ETN, $p<0.05$). In contrast, ethanolamine failed to produce the infarct sparing effect in cardiac specific STAT-3 deficient mice (30.2±2.8% in control vs 29.3±2.6%, $p=ns$). IC, ischaemic control; ETN, ethanolamine; STAT-3, Signal Transducer and Activator of Transcription-3.

4.6. Resveratrol

Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) is a naturally occurring plant polyphenol found in grapes, red wine and peanuts and is touted as having powerful antioxidant, anti-atherogenic and anticancer capabilities (see review, (Juhasz et al., 2010)). Pretreatment with resveratrol was protective against myocardial ischaemia reperfusion injury following infarct size reduction and improved postischaemic ventricular function in rats fed with the alcohol extract (Sato et al., 2002). A study in tumour cells implicated STAT-3 as a target for resveratrol (Yu et al., 2008) led us to investigate and successfully conclude a critical role of this transcription factor in

resveratrol pretreated mice (Figure 10), (Lamont et al., 2011), (I have performed these experiments during my PhD).

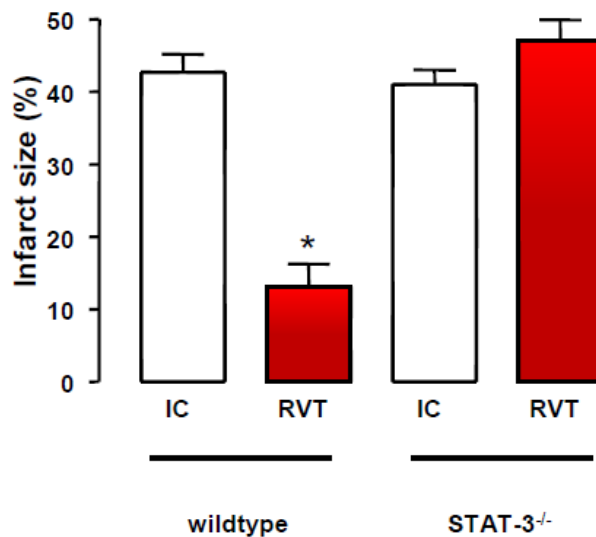


Fig 10. STAT-3 is essential for resveratrol induced preconditioning. Following 30min ischaemia and 45min reperfusion, there was a significantly improved infarct size observed in the resveratrol pretreated hearts compared to the wildtype control ($42.7 \pm 2.5\%$ in control vs $13.1 \pm 3.1\%$, $p < 0.05$). This beneficial effect was not observed in cardiac specific STAT-3 deficient mice ($41.0 \pm 2.0\%$ in control vs $47.0 \pm 2.8\%$, $p = \text{ns}$). IC, ischaemic control; RVT, resveratrol; STAT-3, Signal Transducer and Activator of Transcription-3.

Recently, resveratrol has been shown to activate Silent Information Regulator-1 (SIRT1), a member of the sirtuin family of class III histone deacetylases (Haigis and Sinclair, 2010). SIRT1 has been shown to inhibit apoptosis, protect against oxidative stress in cardiac myocytes and delay the progression of aging in the mouse heart (Alcendor et al., 2004, Alcendor et al., 2007). Transgenic mice with cardiac specific overexpression of SIRT1 were protected against myocardial ischaemia/ reperfusion injury which involved the transcription factor, FoxO1 and upregulation of prosurvival molecules, manganese superoxide dismutase, thioredoxin-1 and Bcl-xL (Hsu et al., 2010). It is possible that resveratrol may induce its cytoprotective effects via both, SIRT1 and STAT-3, though a link has yet to be established.

5.0. The SAFE pathway and conditioning

Recently, a novel prosurvival pathway known as Survivor Activating Factor Enhancement (SAFE) pathway has been identified (Lecour, 2009). It consists of tumour necrosis factor alpha (TNF α) and the Janus Kinase/ Signal Transducer and Activator of Transcription-3 (JAK/ STAT-3) pathway.

5.1 Tumour Necrosis Factor alpha (TNF α) and conditioning

Tumour necrosis factor alpha (TNF α) is a pleiotrophic cytokine that is present in low concentrations in the endothelium and mast cells of healthy hearts (Schulz, 2008). Two types of TNF α receptors have been characterised, namely TNF α receptor 1 (TNFR1, or p55) and TNF α receptor 2 (TNFR2, p75) (Baxter et al., 1999). These receptors are expressed in most cardiac cells, including cardiomyocytes (Kadokami et al., 2000).

Dorge et al. showed that an increased release of TNF α from cardiac cells following persistent ischaemia caused contractile dysfunction (Dorge et al., 2002). In the setting of myocardial ischaemia reperfusion injury, TNF α can cause irreversible injury. Inhibition with TNF α antibodies in dogs and rabbits or blockade of the TNFR1 with soluble TNFR1 given during the pre-ischaemic period reduced infarct size (Belosjorow et al., 2003, Gu et al., 2006, Sugano et al., 2004). Conflicting data has been presented regarding the absolute abolishment of TNF α . One study showed that the infarct size was reduced in TNF α knockout mice compared to the wildtype (Maekawa et al., 2002) but this was refuted in three separate studies (Labruto et al., 2005, Dawn et al., 2004, Flaherty et al., 2008). This suggested that the presence of TNF α may still be required to elicit a protective response. TNFR1 and TNFR2 knockout mouse models (TNFR1^{-/-} and TNFR2^{-/-}, respectively) were engineered to evaluate the role of each receptor in myocardial ischaemia reperfusion injury (Flaherty et al., 2008). Abrogation of TNFR1 (TNFR2, still present) reduced the infarct size after coronary occlusion compared to wildtype and TNFR1^{-/-} mice. This suggested that only TNFR2 was required for protection in this setting.

TNF α knockout mice failed to induce the infarct sparing effect of ischaemic preconditioning (Smith et al., 2002, Ichikawa et al., 2004, Labruto et al., 2005). Although, increased levels of pre-ischaemic TNF α has been observed following classic ischaemic preconditioning, endogenous levels of TNF α were decreased

during the ischaemia-reperfusion injury phase (Belosjorow et al., 1999, Kimura et al., 2006). Exogenous TNF α added and washed out prior to the sustained ischaemic period induced a cardioprotective effect similar to that observed in ischaemic preconditioning (Lecour et al., 2005b). Importantly, protection was restricted to low dose of TNF α (0.5 ng/ml), as higher doses proved to be detrimental. This preconditioning-like protection was also abolished by ROS scavengers (Lecour et al., 2005a) and inhibition of the mitoK_{ATP} channel (Lecour et al., 2002). In the myocardium, mitochondria are responsible for the production of ROS during ischaemia (Di Lisa et al., 2007). The mechanism of activation of ROS by TNF α is unknown but may be present in the mitochondria (Lacerda et al., 2010). The role of TNF α in cardioprotection was further explored when addition of the cytokine to isolated mitochondria, at the protective concentration, improved respiratory parameters following an anoxia-reoxygenation insult (Lacerda et al., 2010). The observed decrease in inner mitochondrial membrane potential was removed in the presense of the mPTP inhibitor, cyclosporine A, therefore suggesting that TNF α may mediate its protective effect via the transient opening of the mPTP.

5.2. Signal Transducer and Activator of Transcription-3 (STAT-3) pathway and conditioning

5.2.1. Activation of STAT-3

The Janus kinases (JAKs) are a family of nonreceptor tyrosine kinases that are located in the cytosol and associate with membrane receptors to initiate signal transduction from the cell to the nucleus (Imada and Leonard, 2000). Structurally, JAKs are composed of seven highly conserved JAK homology domains (JH). They possess a JH1 domain (kinase domain) which is responsible for the catalytic activity of the JAKs; a JH2 domain (pseudo-kinase domain) that is suggested to serve as a docking site for the STATs (Imada and Leonard, 2000); JH3-JH4 region, with a Src Homology 2 (SH2)-like domain but does not have phosphotyrosine binding ability; and the JH4-7 region, which have a FERM domain (four-point-one, ezrin, radixin, moesin) and is involved in JAK-receptor interactions (Figure 11). There are four known JAKs in mammals (JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2)) residing in various cell types but JAK1 and JAK2 are predominant in cardiac

myocytes (Kurdi and Booz, 2007). All of the seven STAT forms are expressed in the heart, i.e. STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. However, STAT1 and STAT3 are the most studied.

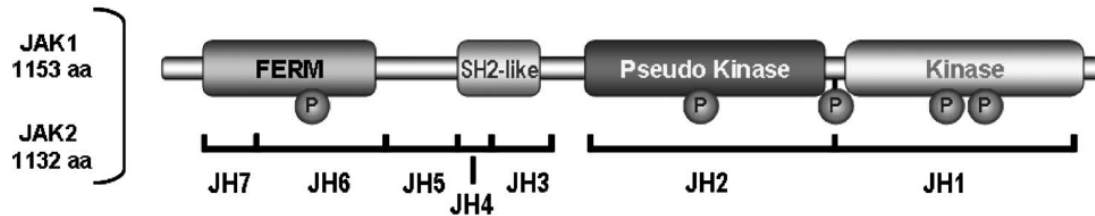


Fig 11. Structure of JAK1 and JAK2 domains. JAK, Janus kinase; JH, JAK homology; FERM, Four-point-one, Ezrin, Radixin, Moesin domain; SH2, Src homology. (from review, (Kurdi and Booz, 2007))

Activation of STAT-1, in association with pro-apoptotic factors such as caspase-1 and p53, is involved in cardiac myocyte induced apoptosis following IR injury (Stephanou et al., 2000b, Stephanou et al., 2001). In contrast, STAT-3 activates anti-apoptotic genes such as Bcl-2 and Bcl-xL (Stephanou et al., 2000a). For the purpose of this thesis, only STAT-3 will be further discussed. The structure of the STAT proteins is conserved within the coiled-coiled domain, DNA binding domain, linker domain and SH2 domain. The transcriptional transactivation domain (TAD) is highly conserved at the carboxyl terminus. The amino terminus is less conserved between the STATs and it may be involved in specific protein responses (Figure 12).

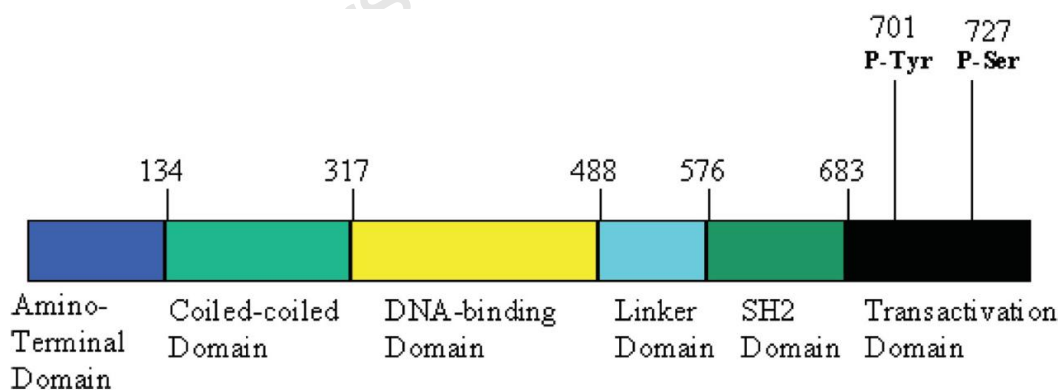


Fig 12. Structure of STAT-3 domain. Refer to text for details. (from review, (Stephanou, 2004))

The JAK2/ STAT-3 pathway may be triggered by the binding of a ligand to a transmembrane receptor leading to the homo- or heterodimerization of the receptor and subsequent phosphorylation and activation of JAK2, associated at the receptor's intracellular domain (Figure 13). Alternatively, the JAK2/ STAT-3 pathway may also

be activated independent of receptor dimerization or association. This has been shown for JAK2 activation in ischaemia/ reperfusion and preconditioning studies where the initiating response is mediated by oxidative stress or G-protein coupled receptors (see review, (Kurdi and Booz, 2009)). JAK2, in turn, phosphorylates the receptor which creates docking sites for the SH2 domain of cytosolic STAT-3. STAT-3 is phosphorylated at its tyrosine-705 residue and forms a homo- or heterodimer by interaction with the SH2 domain of another STAT monomer. The dimers move away from the receptor and translocate into the nucleus where they bind specific DNA sequences and regulate expression of proteins involved in angiogenesis, inflammation, apoptosis, cellular signalling and extracellular matrix composition (Hilfiker-Kleiner et al., 2004, Hilfiker-Kleiner et al., 2005, Snyder et al., 2008). STAT-3 is phosphorylated at its serine-727 residue in its TAD domain which leads to transcription. This transcription can be negatively regulated by Protein Inhibitors of Activated STAT (PIAS) proteins and positively regulated by p300 / Cyclic AMP Response Element Binding (CREB) protein (Wang et al., 2005) and CR-6-interacting factor 1 (CRIF1) (Kwon et al., 2008). STAT-3 proteins are dephosphorylated by nuclear phosphatases and recycled back to the cytosol. Suppressor of Cytokine Signaling (SOCS) genes are also activated by STAT-3 DNA binding and may negatively regulate the JAK2/ STAT-3 pathway by binding to JAK, the receptor or competing with STAT-3 for docking sites on the receptor (see review, (Cooney, 2002)). STAT-3 has been recently identified in the mitochondria but its role and mechanism of action are unclear (Wegrzyn et al., 2009). Mitochondria isolated from STAT-3^{-/-} murine hearts did however, exhibit defects in complexes I and II of the electron transport chain, suggesting a role for STAT-3 as a modulator of mitochondrial respiration.

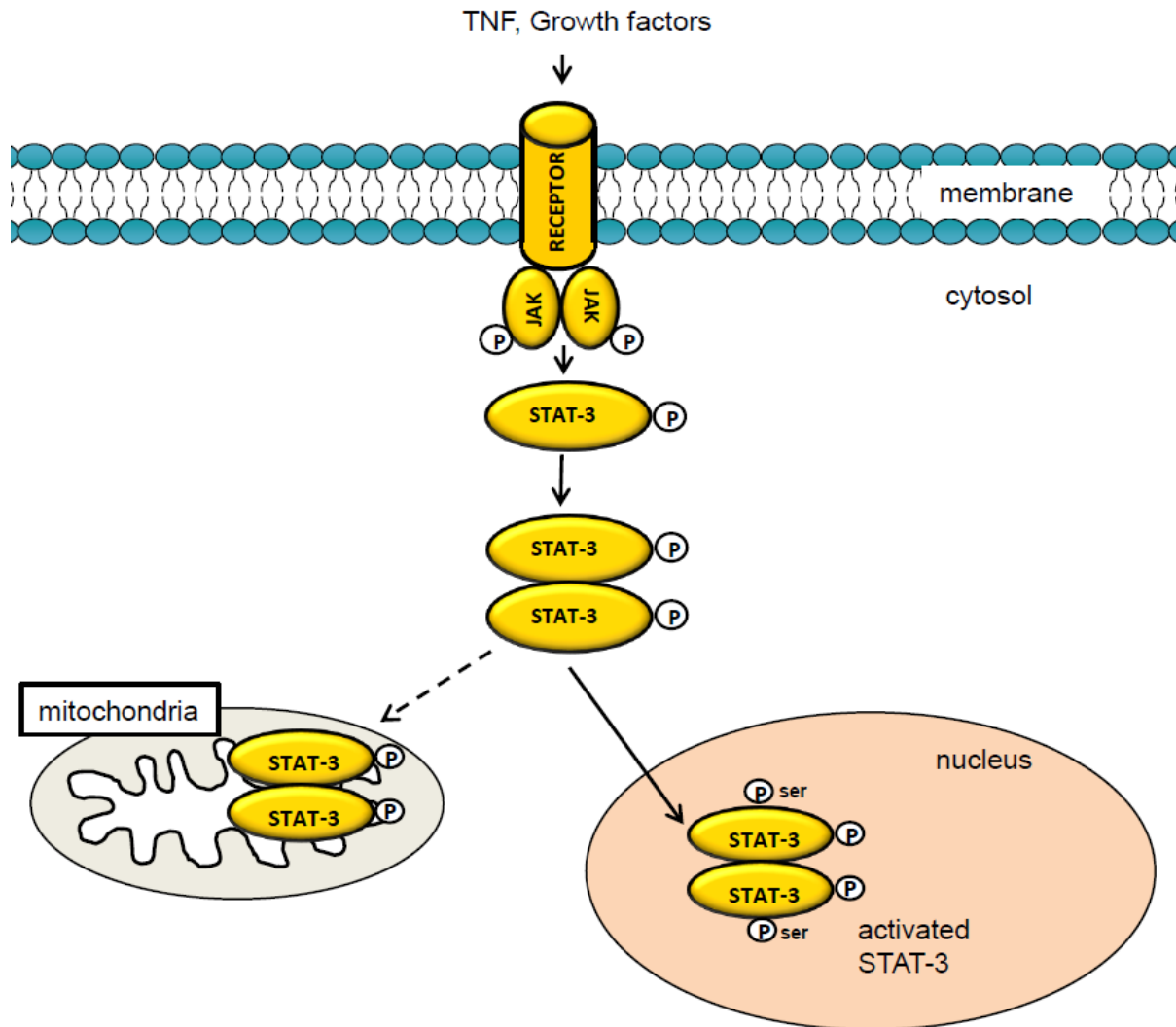


Fig 13. Schematic representation of the JAK/ STAT-3 pathway. Following a stimulus, JAK is recruited to the transmembrane receptor where it becomes phosphorylated. Subsequent dimerization allows JAK to phosphorylate cytosolic STAT-3 at its tyrosine 705 residue. Phosphorylated STAT-3 forms homodimers which translocate to the nucleus where it upregulates transcription and is additionally phosphorylated at its serine 727 residue to enhance DNA interaction. STAT-3 has also been identified in the mitochondria but its role is currently unclear. *JAK*, Janus kinase; *ser*, serine; *Tyr*, tyrosine; *STAT-3*, Signal Transducer and Activator of Transcription-3.

5.2.2. STAT-3 in the heart

The heart is the first organ to form during embryonic development (Foshay et al., 2005). STAT-3 has been shown to be critically important during this phase of life as its genetic ablation led to embryonic lethality (Takeda et al., 1997). However, cardiomyocyte specific STAT-3 deficient (STAT-3^{-/-}) mice are born according to the

Mendelian inheritance ratio and survive to adulthood. They do not exhibit any signs of cardiac dysfunction until 3 months of age where in contrast to the wildtype, they possess reduced capillaries and mild levels of interstitial fibrosis (Jacoby et al., 2003, Hilfiker-Kleiner et al., 2004). However, STAT-3^{-/-} mice become susceptible to heart failure as they progressively age, with increased apoptosis and increased cardiac fibrosis.

5.2.3. STAT-3 in ischaemia/ reperfusion injury

The JAK/STAT-3 pathway was activated via glycoprotein (gp) 130, a signal-transducing membrane receptor in cardiac myocytes following exposure to cytokine, Leukemia Inhibitory Factor (LIF) (Kunisada et al., 1996). The cardiac specific overexpression of STAT-3 was protective against doxorubicin induced cardiomyopathy (Kunisada et al., 2000). Both LIF and an adenovirus-mediated transfection of constitutively activated STAT-3 protected rat neonatal cardiomyocytes against hypoxia/ reoxygenation induced oxidative stress through the upregulation of MnSOD (Negoro et al., 2001). Cardiotrophin-1, another cytokine, also enhanced cell survival and reduced apoptotic cell death in cultured cardiac myocytes by activation of STAT-3 (Stephanou et al., 1998). Furthermore, STAT-3 deficient mice were found to be more susceptible to ischaemic injury and developing heart failure (Hilfiker-Kleiner et al., 2004, Jacoby et al., 2003). These studies suggested an important role for STAT-3 in cardiac survival.

Negoro et al were the first to show the cytoprotective activation of the JAK/ STAT-3 pathway in a rat acute myocardial infarction (AMI). Pretreatment with a JAK2 inhibitor, AG490 before left descending coronary ligation, increased caspase-3 and Bax mediated apoptosis and suppressed the phosphorylation of STAT-3 (Negoro et al., 2000). Cardiac specific mice expressing constitutively activated STAT-3 exhibited significantly reduced infarct size compared to non-transgenic mice and ROS scavengers, metallothionein1 and metallothionein2 were upregulated in the transgenic mice (Oshima et al., 2005). Genetic deletion of these scavengers abrogated the cardioprotective effect of STAT-3.

5.2.4. STAT-3 and preconditioning

Bolli and colleagues demonstrated activation and cytosolic to nuclear translocation of STAT-3 in the in vivo delayed preconditioning of the mouse myocardium (Xuan et al., 2001). Pretreatment with JAK2/ STAT-3 inhibitor, AG490 abolished this protective effect and the upregulation of inducible nitric oxide synthase (iNOS). Hattori et al. showed the activation of JAK2 and STAT-3 in early ischaemic preconditioning in the isolated rat working heart model (Hattori et al., 2001). IPC also induced the recruitment of iNOS and reduced apoptotic cell death with the upregulation of anti-apoptotic gene, bcl-2 and downregulation of proapoptotic gene, bax. Furthermore, the protective effects of classical IPC were not observed in cardiac-specific deficient STAT-3 mice (Smith et al., 2004).

In addition to the pharmacological preconditioning mimetics described earlier, cardiotrophin-1 (Brar et al., 2001), erythropoietin (Rafiee et al., 2005, Nishihara et al., 2006) and cannabinoid agonists (Montecucco et al., 2009) have also been implicated to mediate cardioprotection via activation of STAT-3. Lecour et al demonstrated a critical role for STAT-3 in TNF α induced preconditioning where proapoptotic factor, Bad was inactivated by phosphorylation (Lecour et al., 2005b). Transference of cardioprotection was shown by the treatment of isolated rat hearts with coronary effluent from preconditioned rat hearts resulted in activation of the JAK/STAT-3 pathway (Huffman et al., 2008) that was abolished with AG490. Opioid induced cardioprotection was also abolished with AG490 and reduced the phosphorylation of GSK-3 β , suggesting that STAT-3 may indirectly exert its protective effects on the mitochondria by GSK-3 β mediated inhibition of the mPTP (Gross et al., 2006).

At the time, I started researching my thesis, the role of STAT-3 in ischaemic and pharmacologically postconditioning was unknown and remained to be determined.

6.0. Forkhead box subfamily O (FOXO)

6.1. Definition

The Forkhead helix family of transcription factors are characterized by a conserved DNA binding domain and are critical for early development and adult physiology (Weigel et al., 1989). Several of these FOXO proteins are involved in cardiac

development such as FOXC1, FOXC2, FOXH1, FOXM1, FOXO-1 and FOXP1. FOXO-1, FOXO-3 and FOXO-4 are essential for cardiac function and involved in roles such as response to oxidative stress, regulation of metabolism and apoptosis (Papanicolaou et al., 2008, Kops et al., 2002, Puigserver et al., 2003, Stahl et al., 2002).

6.2 FOXO in the heart

FOXO-1 and FOXO-3a are the most dominant isoforms located in the heart. The nuclear localization sequence of FOXO may be phosphorylated by Akt (Brownawell et al., 2001, Brunet et al., 2002). The phosphorylated FoxO interacts with a subset of 14-3-3 proteins localized in the nucleus, causing translocation of the FOXO-14-3-3 complex to the cytoplasm via the nuclear export protein Crm1. FOXO becomes sequestered by 14-3-3 proteins and is rendered inactive in the cytosol (Brunet et al., 1999, Biggs et al., 1999). Mutation of the Akt phosphorylation sites on FOXO proteins resulted in only FOXO nuclear localization and insusceptibility to Akt-mediated inhibition (Takaishi et al., 1999). The PI3K-Akt signaling is a major regulator of FOXO activity.

Insulin and insulin-like growth factor-1 have the ability to activate Akt and thereby inactivating FOXO by phosphorylation (Matsuzaki et al., 2003). However, sustained FOXO activation by infecting cardiac myocytes with a FOXO-1 adenovirus, created a reverse feedback via increasing Akt levels and subsequently rendering cardiomyocytes insensitive to insulin (Ni et al., 2007). The exact pathophysiological role of FOXO still requires further investigations.

6.3 FOXO and conditioning

The involvement of FOXO in preconditioning has only been described directly as a downstream target of Silent information regulator 1 (Sirt1). Sirt1 belongs to the sirtuin family class III histone deacetylases and thus require NAD⁺ for their enzymatic activity (Imai et al., 2000). Sirt1 are involved in activities such as retarding aging and protecting the heart against oxidative stress (Alcendor et al., 2004, Alcendor et al., 2007). Rats treated with resveratrol, a phenolic compound found on the skin of

grapes, activated Sirt1 and phosphorylated FOXO-1 in the heart (Mukherjee et al., 2009). This resulted in a decreased infarct size and reduced apoptosis when isolated hearts were subjected to ischaemic injury. Sirt1 was also shown to inhibit oxidative stress by upregulation of mnSOD via FOXO-1 during ischaemia reperfusion (Hsu et al., 2010). The exact role of FOXO-1 in pre- and postconditioning has yet to be determined.

University of Cape Town

B. AIM, HYPOTHESIS AND OBJECTIVES

1.0 Aim

Ischaemic postconditioning is a phenomenon whereby brief episodes of intermittent ischaemia and reperfusion applied immediately following a prolonged ischaemic event significantly reduces infarct size and improves cardiac function. This profound effect has been translated across different species although the exact signalling mechanism has yet to be determined, as this may yield novel therapeutic opportunities against ischaemic heart disease. In preconditioning, two prosurvival pathways have been identified. The first was the Reperfusion Injury Salvage Kinase (RISK) pathway which involved the activation of prosurvival kinases, phosphatidylinositol 3-kinase (PI3-Kinase)/ Akt and extracellular signal-regulated kinase (ERK 1/2) which has been well established and documented in cardioprotective studies. More recently, the Survival Activating Factor Enhancement pathway has been discovered that involved the activation of Tumour Necrosis Factor- α (TNF α) and Signal Transducer and Activator of Transcription-3 (STAT-3).

Pharmacological studies with preconditioning agents such as adenosine, bradykinin, opioids and sphingosine 1-phosphate (S1P) have been shown to successfully confer protection and implicate some of these and downstream factors. Postconditioning, however, is a relatively new discovery. The prosurvival kinases of the RISK pathway and the mitochondria have been implicated but very little is known about the involvement of the SAFE pathway. STAT-3 is a transcription factor that is associated with cell growth, proliferation and survival. The aim of this present study was to investigate the role of STAT-3 in relation to the SAFE pathway in ischaemic and pharmacological postconditioning induced cardioprotection.

1.1 Hypothesis and Objectives

We hypothesized that activation of STAT-3, as part of the SAFE pathway, is essential in both ischaemic and pharmacological postconditioning to confer cardioprotection (Figure 14).

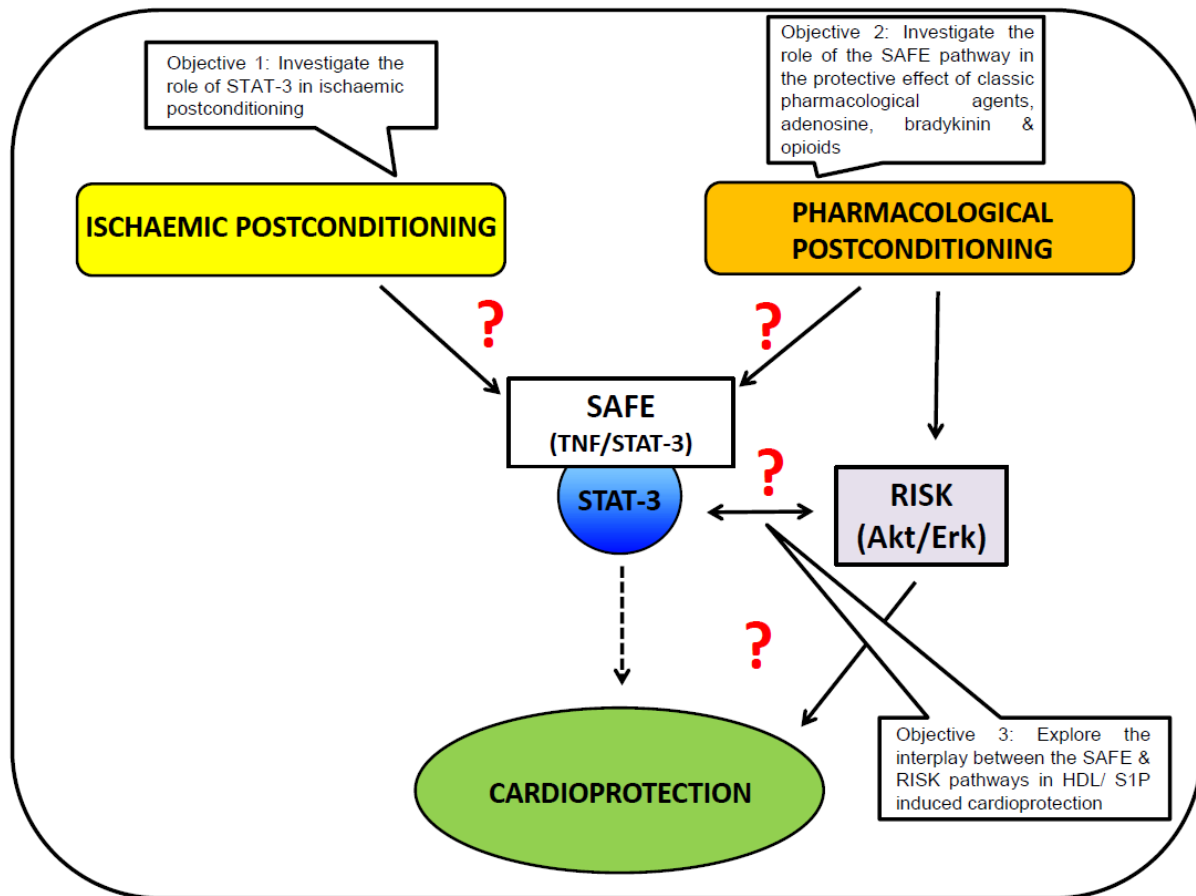


Fig 14. Schematic representation of hypothesis and objectives in ischaemic and pharmacological postconditioning. The JAK/STAT-3 pathway may be stimulated by ischaemic or pharmacological postconditioning. Abbreviations as defined in text.

To fulfil this aim, we set the following objectives:

1) To investigate the role of STAT-3 in ischaemic postconditioning

To achieve this objective we will employ the use of cardiac specific STAT-3 deficient (STAT-3^{-/-}) mice and their respective wildtype. Isolated wildtype hearts will be postconditioned to optimise the parameters suited for this model. STAT-3^{-/-} hearts will be subjected to ischaemic postconditioning. Should STAT-3 prove necessary in IPostC induced cardioprotection, we will use the western blot technique to confirm its role and examine GSK-3 β as a possible downstream target of STAT-3.

2) To investigate the role of STAT-3 with classic pharmacological postconditioning agents

For this study, we will use TNF deficient mice (TNF α ^{-/-}), and cardiac specific STAT-3 deficient mice and their respective wildtypes. Isolated hearts will be subjected to an ischaemia reperfusion insult and classic pharmacological agents, adenosine, bradykinin and opioids will be used to mimic the postconditioning effect.

3) To investigate the role of STAT-3 in HDL/ S1P induced cardioprotection and to delineate a possible interplay between the SAFE and RISK pathways to protect the heart

To achieve this objective we will use TNF deficient (TNF α ^{-/-}), cardiac specific STAT-3 deficient (STAT-3^{-/-}) mice and their respective wildtypes. HDL will be extracted from the blood of healthy patients. Isolated hearts will be subjected to an ischaemia reperfusion insult and perfused with either HDL or S1P to confer a protective effect similar to postconditioning. Should STAT-3 prove necessary, we will examine the possible interplay between the SAFE and RISK pathways by using inhibitors of various pathways and the western blot technique to evaluate Akt, ERK1/2, STAT-3 and FOXO-1 levels.

C. MODELS AND METHODS

1.0. Genetically modified animals

All the animal experiments conducted with approved by the Animal Research Ethics Committee of the University of Cape Town, and followed the recommendations stipulated in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No.85 (23), revised 1996).

1.1. Mouse models to investigate the role of STAT-3 in cardiac protection

Homozygous TNF α -deficient (TNF $\alpha^{-/-}$) mice and their respective wildtype, Black 6 x 129S (TNF-WT) were a generous gift from Dr Muazzam Jacobs and Professor Bernhard Ryffel, Department of Immunology, University of Cape Town.

Cardiomyocyte specific STAT-3- deficient (STAT-3 $^{-/-}$) mice and their respective wildtype, were based on a C57 Black 6 background and created in our facilities by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre recombinase mice, as previously described (Smith et al., 2004). All mice were 14-16 weeks of age, unless where indicated otherwise.

1.1.1. Creation of cardiomyocyte specific STAT-3 deficient mice

Early attempts to create a whole body STAT-3 deficient mouse were unsuccessful as it resulted in the degeneration of mouse embryos between days 6.5 and 7.5 of gestation (Takeda et al., 1997). This indicated the STAT-3 was expressed early in life and critical for the embryonic development. For the purposes of our investigations, we undertook the creation of a conditional, cardiac specific STAT-3 knockout mouse. This was achieved with the Cre-Lox P system of conditional gene ablation.

1.1.2. Cre Lox P technology

This system of conditional gene targeting employed two separate transgenic gene lines; a homozygous STAT-3 floxed mouse (STAT-3 $^{F/F}$; F-Floxed) and an organ specific heterozygous Cre recombinase-expressing mouse (Cre $^{T/N}$; T – Transgene and N – Normal). These 2 strains of mice were then crossed to produce the organ specific STAT-3 KO animal (Cre $^{T/N}$ STAT-3 $^{F/F}$).

1.1.3. STAT-3 floxed mouse

Floxed mice are animals that have a specific DNA sequence, Lox P (Locus of X-ing over P) site inserted and integrated into their germ line. Two Lox P sites have been inserted in the DNA sequence of the protein to be disrupted such that the target segment is flanked by each Lox P fragment. Each Lox P site contains two 13 base pair repeats separated by an 8 base pair asymmetric spacer region (Figure 15). Lox P sites arranged in an opposite orientation to each other, invert the DNA between them (Figure 16A). In contrast, Lox P sites arranged in a direct orientation act as targets for excision of the DNA between them (Figure 16B). Heterozygous mice possessing Lox P sites integrated into the STAT-3 germ line ($Cre^{N/N}$ STAT-3^{F/N}) in a direct orientation (Figure 16B) were obtained from Professor S Akira, Osaka University, Japan (Takeda et al., 1997).

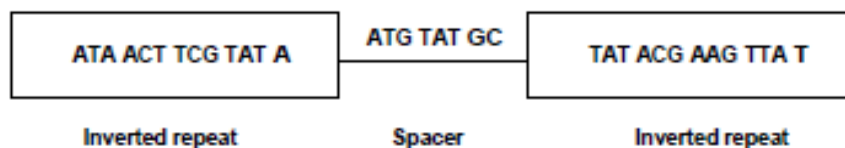


Fig 15. Lox P site. DNA sequence of a Lox P site which is made up of 2, 13 base pair inverted repeats separated by an 8 base pair asymmetric spacer region. DNA, Deoxyribonucleic Acid.

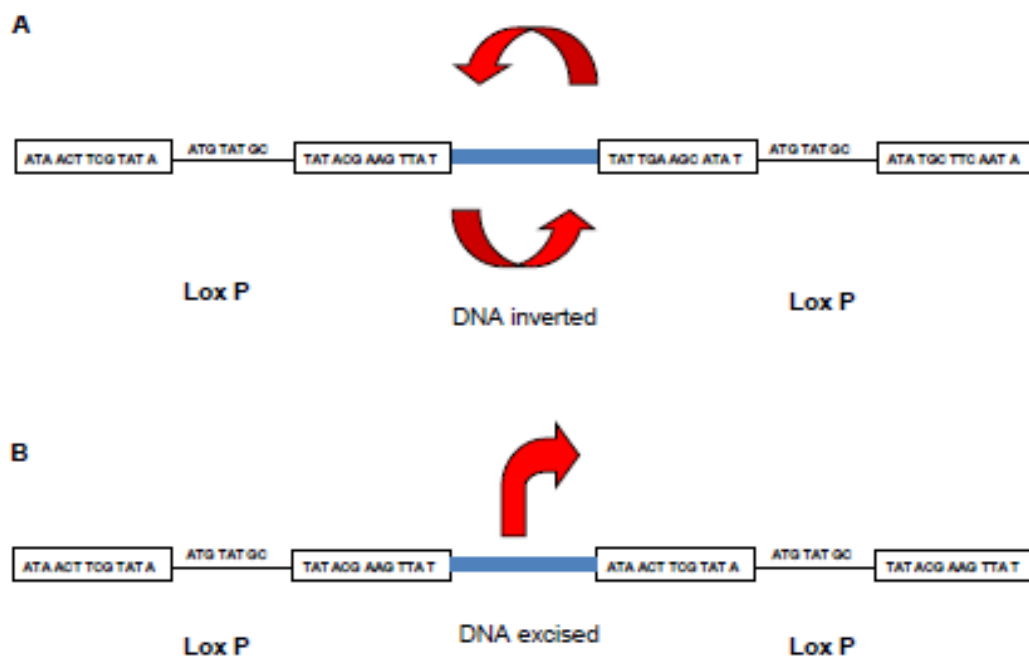


Fig 16. Functional orientation of Lox P sites. The orientation in which Lox P sites are integrated into the genome determines whether the DNA is inverted as in (A) or excised as in (B). DNA, Deoxyribonucleic Acid; Lox P, Locus of X-ing over P site.

1.1.4. Cardiac specific expressing Cre Recombinase mouse

The cardiac specific Cre recombinase expressing mouse used to generate our STAT-3 Knockout animal was a generous gift from Professor Kenneth R Chien of Harvard Medical School. The DNA between the Lox P sites is excised by the expression of the 38 kDa bacteriophage P1 Cre recombinase protein (Chien, 2001). This enzyme cleaves the sequence, removing the DNA and leaving behind one Lox P site (Figure 17C). The expression of the Cre enzyme was placed under the control of a tissue specific promoter for cardiomyocytes. This ensured that the Cre recombinase would only be expressed in the cardiac tissue and thus specifically target deletion of the floxed sequence. A promoter with a high transcriptional activity had to be chosen that would result in a prominent expression of the Cre recombinase protein and lead to a high rate of Lox P site excision. To this end, the Myosin Light Chain 2 Ventricle (MLC2V) promoter was selected (Chen et al., 1998). The Cre recombinase was placed on one of the alleles (allele – one of 2 forms of the same gene), under control of the MLC2V promoter. This promoter is only active in the ventricles of adult mice and is highly expressed ensuring an elevated expression of Cre recombinase.

Lox P sites were introduced into introns 21 and 22 (non-coding regions of DNA) which flanked the tyrosine phosphorylation site of exon 21 (coding region of DNA) (Figure 17B). Removal of exon 21 (Figure 17B) by Cre recombinase will result in the transcription and translation of a truncated non-functional STAT-3 protein (Figure 17C).

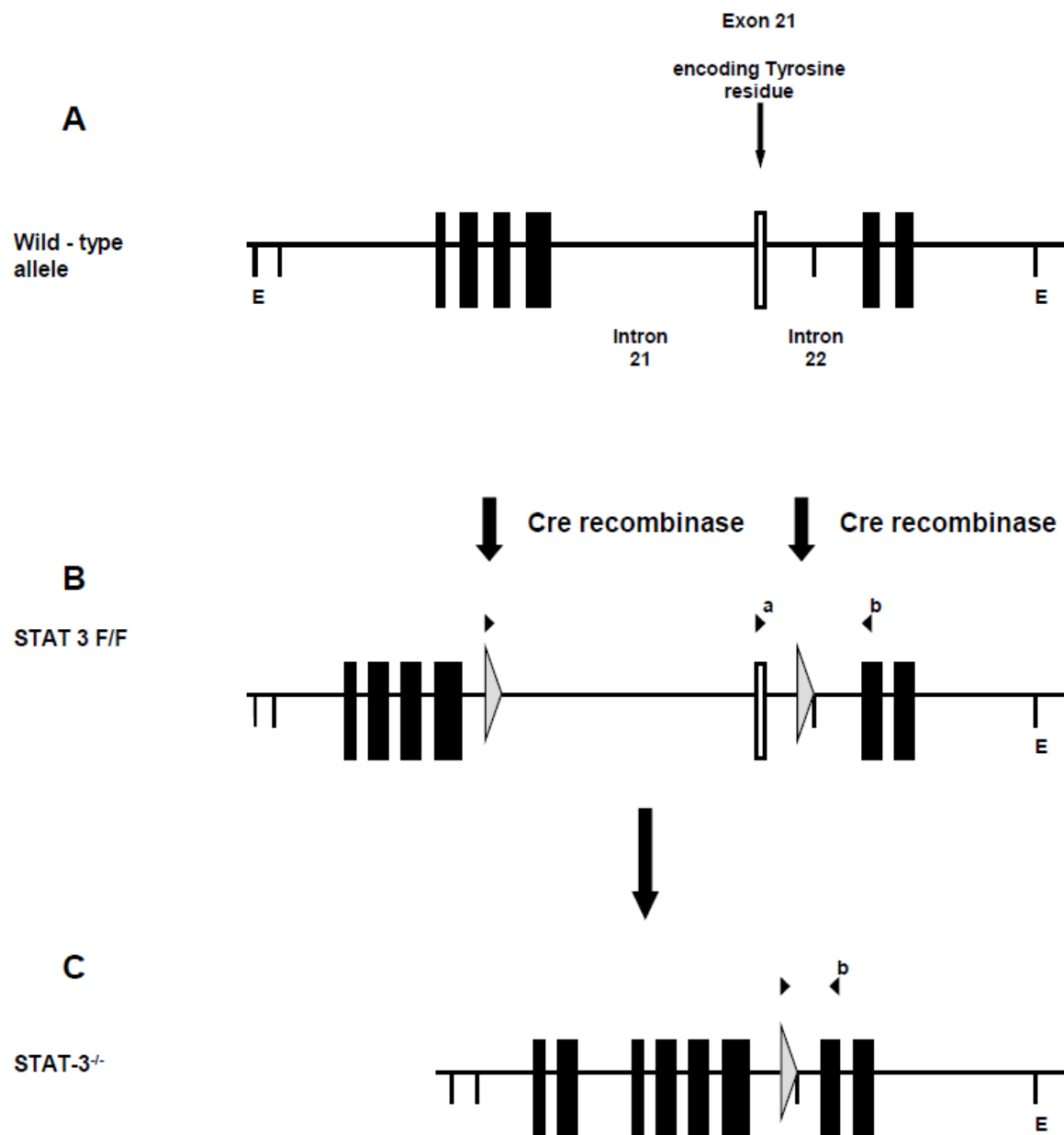




Fig 17. Targeted deletion of STAT-3. Gene organisation in STAT 3 ^{F/F} mice before and after Cre mediated disruption. () Exon 21 of STAT 3 was flanked by identical lox P () sites (B). Each site is a target for Cre recombinase which is used to disrupt the STAT 3 gene, effectively knocking STAT 3 out. "a" and "b" are the primers used to determine whether the mouse is harboring a floxed STAT 3 gene. Abbreviations are as defined in text.

1.1.5. Breeding strategy for the establishment of a cardiac specific STAT-3 deficient mouse colony

The cardiac specific STAT-3 knockout with littermate controls were bred according to Figure 18. The original pairs of STAT-3 floxed mice had no Cre insert and were heterozygous for the STAT-3 floxed allele ($\text{Cre}^{\text{N/N}}$ STAT-3^{F/N}). These mice were bred together and the genotypes generated were calculated as in the Punnett square of Figure 19A and the subsequent genetic distribution of genotypes in Figure 19B. The $\text{Cre}^{\text{N/N}}$ STAT-3^{F/F} mice were selected for establishing a stable breeding colony.

The $\text{Cre}^{\text{N/N}}$ STAT-3^{F/F} mice were crossed with the transgenic Cre recombinase expressing strain ($\text{Cre}^{\text{T/N}}$ STAT-3^{N/N}) to generate $\text{Cre}^{\text{T/N}}$ STAT-3^{F/N} mice (Figure 20A). Cross breeding these mice gave us two distinct genotypes, $\text{Cre}^{\text{T/N}}$ STAT-3^{F/N} and $\text{Cre}^{\text{N/N}}$ STAT-3^{F/N} mice (Figure 20A) in a 50/50 ratio (Figure 20B). From this result, $\text{Cre}^{\text{T/N}}$ STAT-3^{F/N} mice were selected and crossed with $\text{Cre}^{\text{N/N}}$ STAT-3^{F/F} mice (Figure 19B and Figure 21) to spawn $\text{Cre}^{\text{T/N}}$ STAT-3^{F/F} mice, the desired genotype of cardiac specific STAT-3^{-/-} mice. This breeding approach also gave rise to three other distinct genetic backgrounds (Figure 21A) with the STAT-3^{-/-} mice making up a quarter (Figure 21B) of the offspring.

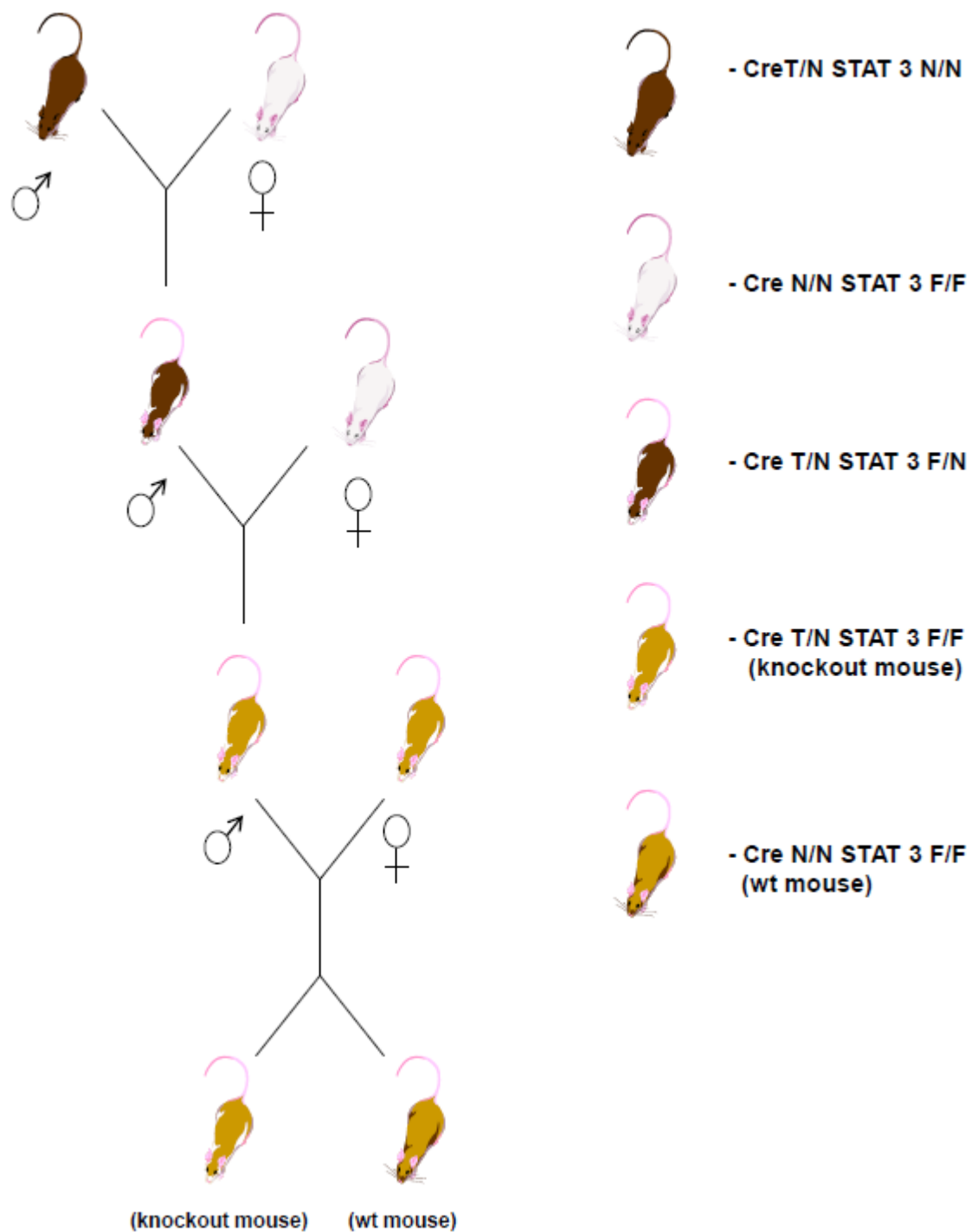


Fig 18. Generation of ventricle specific knockout mice. *N*, normal; *T*, Transgenic Cre insert; *F*, Floxed STAT-3. Of note is that Cre^{T/T} is embryo lethal due to the absence of MLC2V. Here no offspring can be Cre^{T/T}.

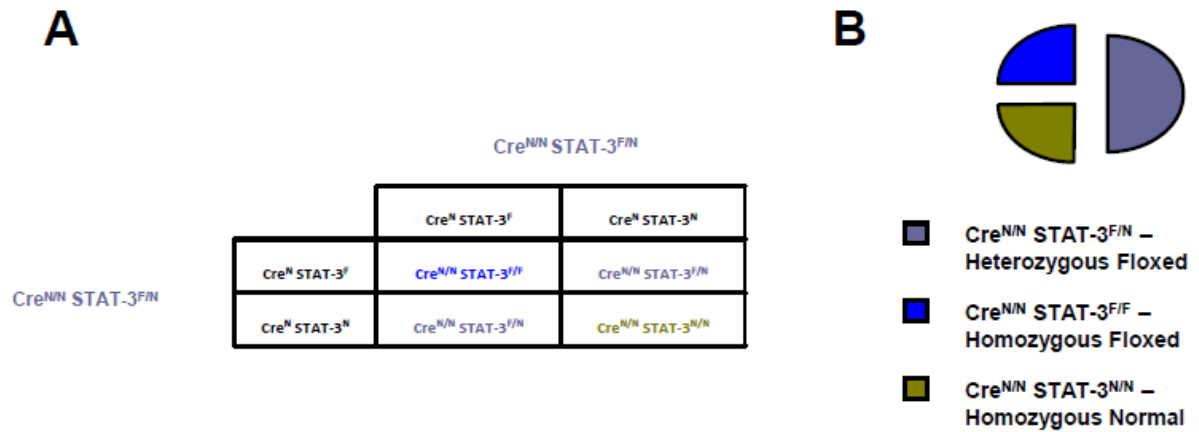


Fig 19. Mouse Breeding: $Cre^{N/N} STAT-3^{F/N}$ with $Cre^{N/N} STAT-3^{F/N}$. The punnett square (A) is used to illustrate the genetic distribution of the floxed allele in offspring of $Cre^{N/N} STAT-3^{F/N}$ parents. Crossing heterozygous floxed mice ($Cre^{N/N} STAT-3^{F/N}$) resulted in 3 distinctive genotypes. Homozygous floxed mice ($Cre^{N/N} STAT-3^{F/F}$), heterozygous floxed mice ($Cre^{N/N} STAT-3^{F/N}$) and homozygous normal ($Cre^{N/N} STAT-3^{N/N}$) mice. The resulting Mendelian ratios of the offspring is shown in B. Abbreviations are as in text.

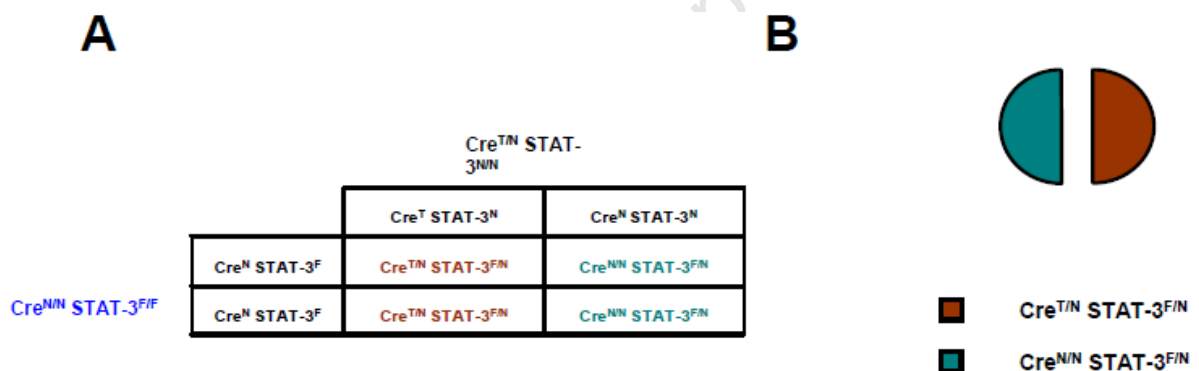


Fig 20. Mouse Breeding: $Cre^{N/N} STAT-3^{F/F}$ with $Cre^{T/N} STAT-3^{N/N}$. Cross breeding $Cre^{N/N} STAT-3^{F/F}$ and $Cre^{T/N} STAT-3^{N/N}$ gave 2 variant mouse strains (A), $Cre^{T/N} STAT-3^{F/N}$ as well as $Cre^{N/N} STAT-3^{F/N}$ mice in equal ratios (B). Abbreviations are as in text.

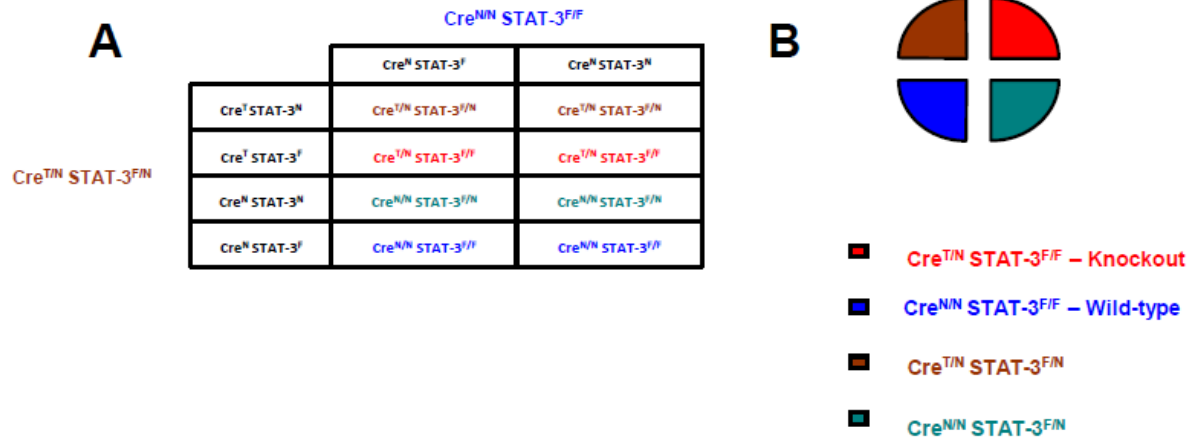


Fig 21. Mouse Breeding: Cre^{T/N} STAT-3^{F/N} with Cre^{N/N} STAT-3^{F/F}. Cross breeding Cre^{T/N} STAT-3^{F/N} and Cre^{N/N} STAT-3^{F/F} mice (A) gave rise to 4 (B) distinct genotypes of which 2, Cre^{T/N} STAT-3^{F/N} and Cre^{N/N} STAT-3^{F/N} were discarded as they did not possess the correct genetic configuration for both wild-type or STAT-3 KO mice. Abbreviations are as in text.

Cre^{T/N} STAT-3^{F/F} mice were selected and used to create and maintain a stable breeding population of STAT-3^{-/-} and littermate control mice (Figure 22A). The Mendelian STAT-3^{-/-} to wild-type ratio of this breeding strategy produced 25% wild-type (Cre^{N/N} STAT-3^{F/F}) and 50% STAT-3^{-/-} (Cre^{T/N} STAT-3^{F/F}) mice (Figure 22B). In addition, 25% of the embryos were homozygous for the Cre insert, i.e., Cre^{T/T} STAT-3^{F/F} which was embryo lethal, as both alleles of the MLC-2V gene were replaced, and effectively deleted this critical gene (Chen et al., 1998).

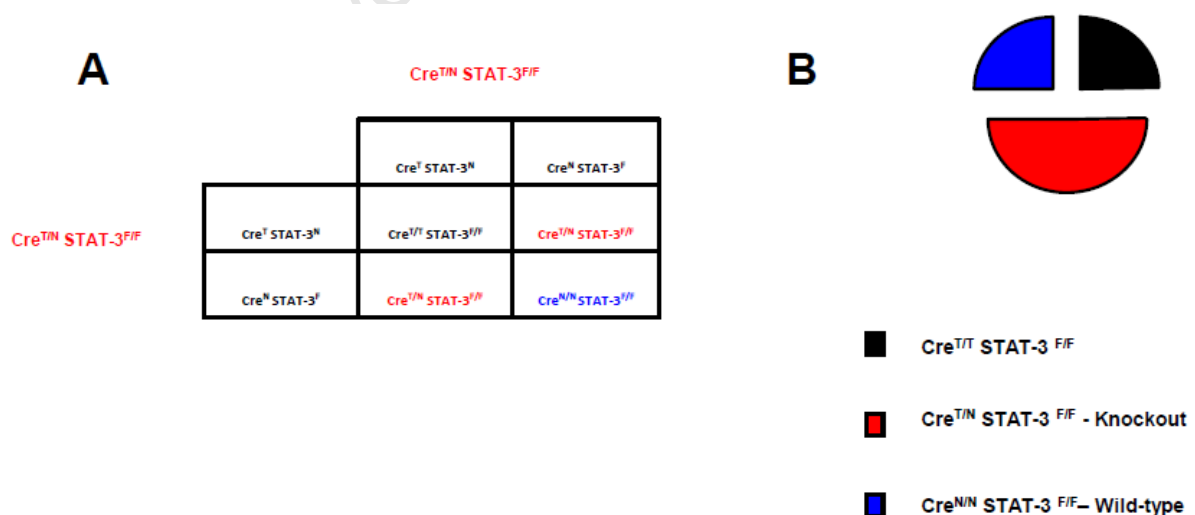


Fig 22. Mouse Breeding: Cre^{T/N} STAT-3^{F/F} with Cre^{T/N} STAT-3^{F/F}. The breeding strategy used to maintain a stable mouse colony is illustrated in (A). The offspring ratio is shown in (B). Abbreviations are as in text.

1.2. Genotyping

Since the mice were phenotypically similar, genotyping was employed to differentiate the $\text{TNF}\alpha^{-/-}$ and $\text{STAT-3}^{-/-}$ from their respective wildtype. Genomic DNA was extracted from mouse tail cuts sourced at weaning (3 weeks of age). Genotyping was carried out by means of polymerase chain reaction (PCR).

1.2.1. DNA Extraction

Approximately 1 cm of mouse tail was excised and digested overnight at 55°C in 400 µl tail digestion buffer (0.5 M Tris pH 8.0, 0.1 M EDTA, 1% SDS) with 1 mg/ml proteinase K (Roche, Mannheim) in 1.5 ml micro-centrifuge tubes. Each tube containing digested sample was then treated with 400 µl of Phenol:Chloroform:Alcohol (25:24:1) (Sigma, USA) mixture and gently inverted to mix. Samples were centrifuged at 13,500 rpm (Heraeus MK202, Germany) for 10 minutes at room temperature to remove the cellular debris by density separation. The bottom layer containing the debris was carefully removed and discarded with a sterile barrier tipped pipette. Phenol traces were removed from the DNA with the addition of 200 µl of chloroform (Sigma, USA) to each tube and gentle inversion prior to centrifugation at 13,500 rpm for 5 minutes. Two layers are formed in each tube, isolating the DNA into the top layer. Once again, the bottom layer is gently discarded with aid of sterile barrier tips. The tubes containing the top phase were centrifuged at 13,000 rpm for 10 minutes, pelleting any remaining debris. After transferring the liquid phase into a new sterile 1.5 ml micro-centrifuge tube, 50 µl of 7.5 M ammonium acetate (Merck) and 1ml 100% ethanol was added to each tube and gently inverted to mix. DNA was visible at this point, as a white stringy precipitate. The DNA solution was transferred to another clean micro-centrifuge tube containing 500 µl of 70 % ethanol to hydrate the DNA and centrifuged at 13,000 rpm for 10 minutes. The ethanol was carefully decanted taking care not to discard DNA settled at the base of the tube. DNA was air dried to evaporate the remaining ethanol and then dissolved in 50-75 µl of ultra pure RNase free water. Samples were stored at -20°C until PCR.

1.2.2. Polymerase Chain Reaction (PCR)

TNF α ^{-/-} mice were generated by the insertion of a phosphoglycerate kinase-neomycin expression cassette within the TNF α gene. This technique of targeted gene deletion and detection has been previously described (Marino et al., 1997). Genotyping of the TNF α gene was randomized to confirm the genotyping characteristics of the mice. In contrast, cardiac specific STAT-3 deficient mice were genotyped at each stage of the breeding process since the offspring could be both STAT-3^{-/-} and wildtype. Hence, candidate STAT-3^{-/-} mice were screened for the presence of Cre recombinase insertion and floxed alleles.

1.2.3. Sequences and PCR for TNF α ^{-/-}

a) TNF^{-/-}: Sense primer: 5'-CTA AAT GAA CTG CAG GAC GA-3'

Anti-sense primer: 5'-ATA CTT TCT CGG CAG GAG CA-3'

Neomycin: Sense primer: 5'-CTG AAT GAA CTG CAG GAC GA-3'

Anti-sense primer: 5'-ATA CTT TCT CGG CAG GAG CA-3'

PCR reactions were performed in a volume of 50 μ l:

10x Magnesium free buffer:	10.0	μ l
4 mM MgCl ₂ :	8.0	μ l
100 μ M dNTP's:	1.6	μ l
Primer (TNF or Neo):	0.5	μ l
β actin 3':	0.5	μ l
β actin 5':	0.5	μ l
H ₂ O:	26.9	μ l
Taq:	0.5	μ l

DNA sample: 1.5 μ l

50.0 μ l

PCR Conditions:

TNF α :

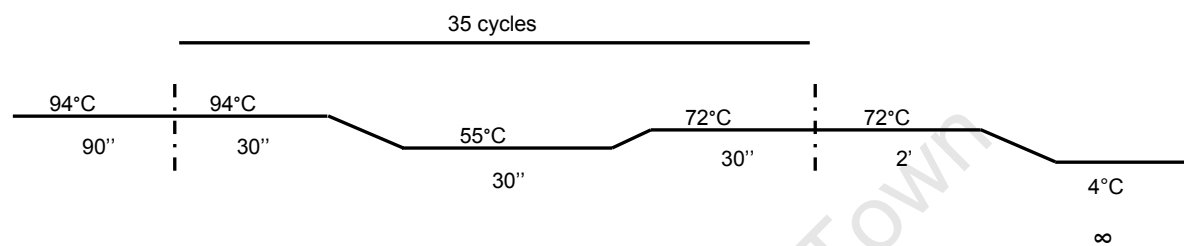


Fig 23. PCR conditions for TNF α genotyping

Neo:

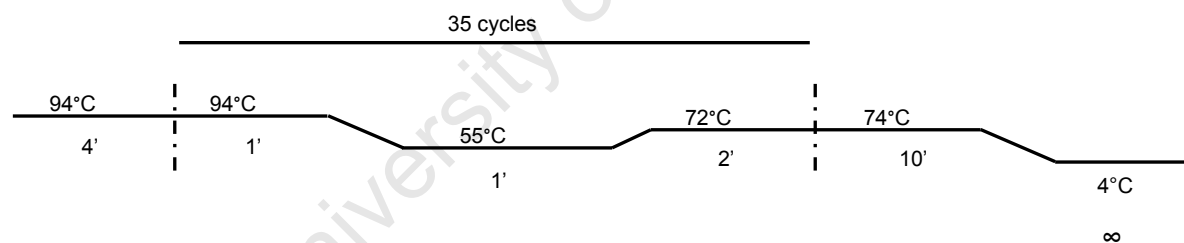


Fig 24. PCR conditions for Neo genotyping

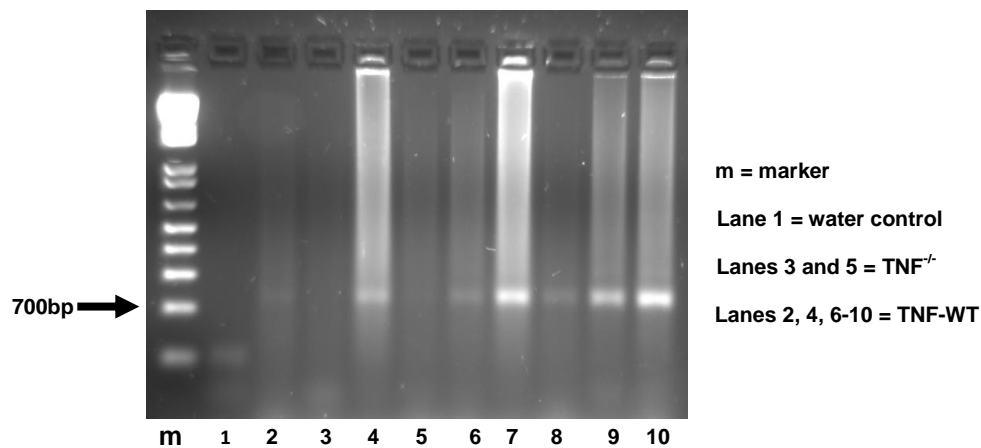


Fig 25. UV gel analysis revealing the targeted TNF gene deletion from mouse genomic DNA

1.2.4. Sequences and PCR for cardiac specific STAT-3^{-/-} mouse model

Cre positive mice were identified with the following sequences:

Cre Sense primer: 5'-GTT CGC AAG AAC CTG ATG GAC A-3'

Cre Anti-sense primer: 5'-CTA GAG CCT GTT TTG CAC GTT C-3'

The presence of the Myosin Light Chain Isoform-2 Ventricle (MLC2V) gene was included as a positive control as it is exclusively expressed in the heart.

MLC2V Sense primer: 5'-GGC AAC CCT CAG ACA CCA T-3'

MLC2V Antisense primer: 5'-TGT GGA GGC TCT GGA TCA GGA C-3'

The PCR reactions were performed in 50 µl volumes as follows:

10x Magnesium free buffer:	10.0	µl
4 mM MgCl ₂ :	8.0	µl
100 µM dNTP's:	1.6	µl
Primer:	0.5	µl
β actin 3':	0.5	µl

β actin 5':	0.5	μ l
H ₂ O:	26.9	μ l
Taq:	0.5	μ l
DNA sample:	<u>1.5</u>	μ l
	50.0	μ l

PCR Conditions:

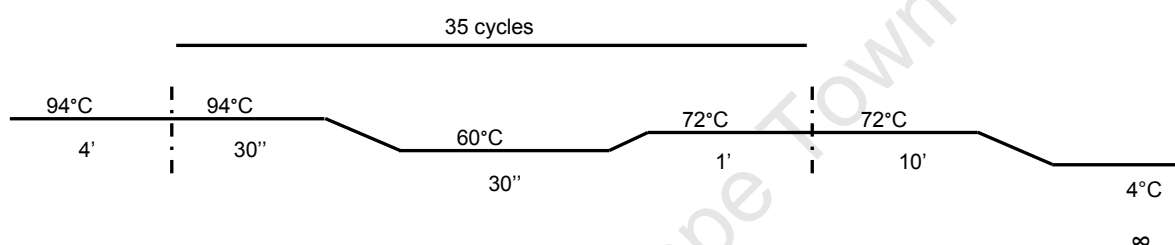


Fig 26. PCR conditions for STAT-3^{-/-} genotyping

Floxed STAT-3 gene segments yielded a 250 bp fragment on the gel. A 200 bp fragment was observed if STAT-3 gene was not floxed. Heterozygous mice were identified by the presence of a double band. STAT-3 floxed and non-floxed DNA were amplified with the following primers:

STAT-3 primer (a) 5'-CCT GAA CAA GTT CAT CTG TGT GAC-3'

STAT-3 primer (b) 5'-CAC ACA AGC CAT ACT CTG GTC TCC-3'

The PCR reactions were performed in 50 μ l volumes as follows:

10x Magnesium free buffer:	5.0	μ l
4 mM MgCl ₂ :	4.0	μ l
100 μ M dNTP's:	0.8	μ l

Primer:	0.25 μ l
β actin 3':	0.25 μ l
β actin 5':	0.25 μ l
H ₂ O:	13.2 μ l
Taq:	0.25 μ l
DNA sample:	<u>1.0 μl</u>
	25.0 μ l

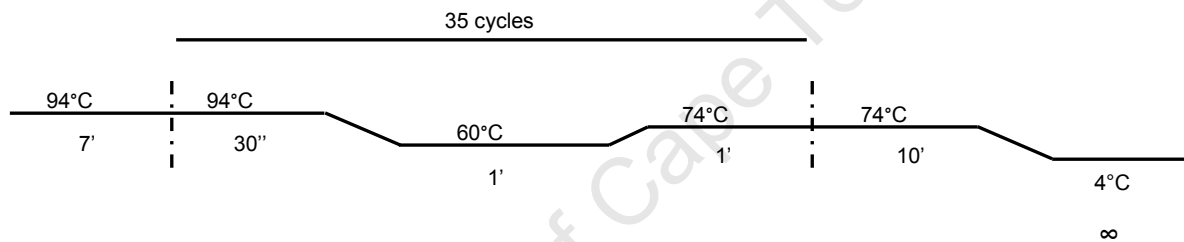


Fig 27: PCR conditions for STAT-3 floxed mice.

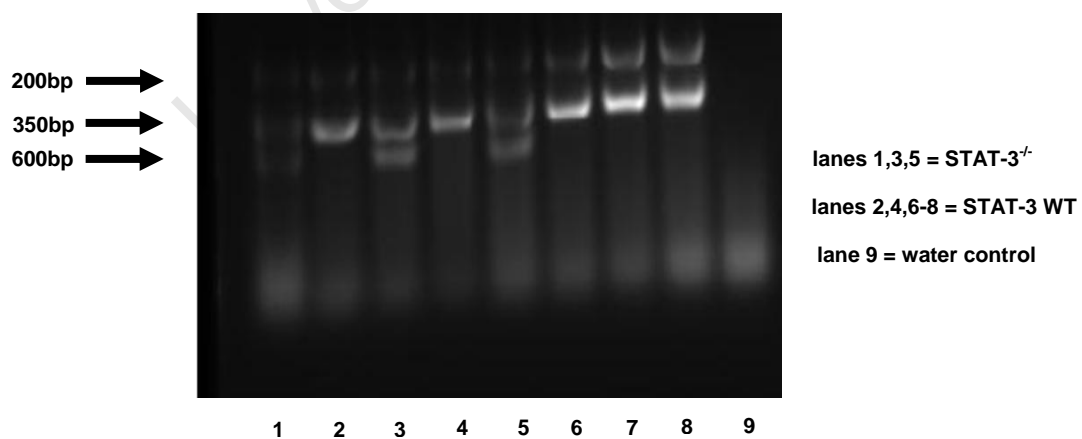


Fig 28. UV gel analysis showing Cre positive band (350 bp) and the control MLC2V band at 600 bp. MLC2V, Myosin Light Chain Isoform-2 Ventricle.

1.3. Agarose gel Electrophoresis

The PCR product was separated on a 2% agarose gel. 100 ml of Tris Acetate electrophoresis buffer (TAE) (0.04 M Tris-Acetate, 0.001 M EDTA) was added to 2 grams of agarose (Whitehead Scientific, RSA) and heated in a microwave oven at 70 % power until dissolved. It was possible to visualize the amplified DNA fragments under ultra-violet (UV) by the addition of 1 μ l ethidium bromide per 100 ml agarose gel. The agarose solution was poured into the gel mould and allowed to set for 15 minutes. TAE buffer was added to gel apparatus prior to removal of the gel combs. Each well was loaded with 10 μ l of PCR product and 2 μ l of gel lading buffer (0.25% Bromophenol Blue-Xylene Cyanole Dye Solution, Sigma). 4 μ l of a 100 bp DNA ladder marker (Roche, Germany) was also loaded on to the gel and ran concurrently with the samples at 90 V for 30 - 40 minutes. The bands were visualized on a transilluminator (UviTec, USA). Gel photos of the PCR were recorded using the UviTec Geldoc apparatus with UViband software v.97 (Cambridge, UK).

2.0. Isolated mouse heart model

Adult male $\text{TNF}\alpha^{-/-}$ and cardiomyocyte-specific $\text{STAT-3}^{-/-}$ mice and their respective wildtypes were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and heparin (25 IU ip). The pedal reflex test was implemented to confirm an adequate level of anaesthesia prior to the opening of the chest. The sternum and attached costal cartilage was excised to provide access to the mediastinum. Hearts were rapidly excised and placed in cold arrest Krebs-Henseleit solution (NaCl 118.0 mM; NaHCO_3 24.0 mM; KCl 4.0 mM; NaH_2PO_4 1.0 mM; CaCl_2 2.5 mM; MgCl_2 1.2 mM; di-sodium EDTA 0.5 mM; glucose 10.0 mM; gassed with 95% O_2 /5% CO_2 at 37°C) before mounted on a Langendorff retrograde perfusion apparatus. Pressure was kept constant at 110 cmH_2O and temperature readings were acquired by means of a fine thermocoupled wire (Physitemp, NJ, USA), inserted into the right atrium and monitored on a Digitron 2600T temperature sensor (Torquay, UK). Developed tension was assessed in the hearts by the application of a rigid lightweight lexan coupling rod connected to a force placement transducer (Grass FT03C, MA, USA). A 4-0 silk attached to a 20 mm curved atraumatic needle was threaded through the apex of the heart and firmly fastened to

the coupling rod. Diastolic tension was adjusted to a 2 g weight and hearts were paced to 560 bpm. Tension output was recorded on a Powerlab Labchart recorder (ADI instruments, Australia). Coronary flow rate was measured at timed intervals. A minimum flow rate of 1.5 ml/min and maximum flow of 5.0 ml/min of coronary flow rate, heart rate between 460 - 600 bpm and developed force ≥ 4 was deemed acceptable. These parameters (coronary flow, heart rate, and developed force) served as a measure of cardiac function. No haemodynamic data was collected. Isolated hearts were allowed to stabilize for 25 minutes prior to 35 minutes global ischaemia (achieved by means of a stopcock to prevent further flow of buffer into the heart from the aortic cannula) and a 45 minute reperfusion period (Smith et al., 2002).

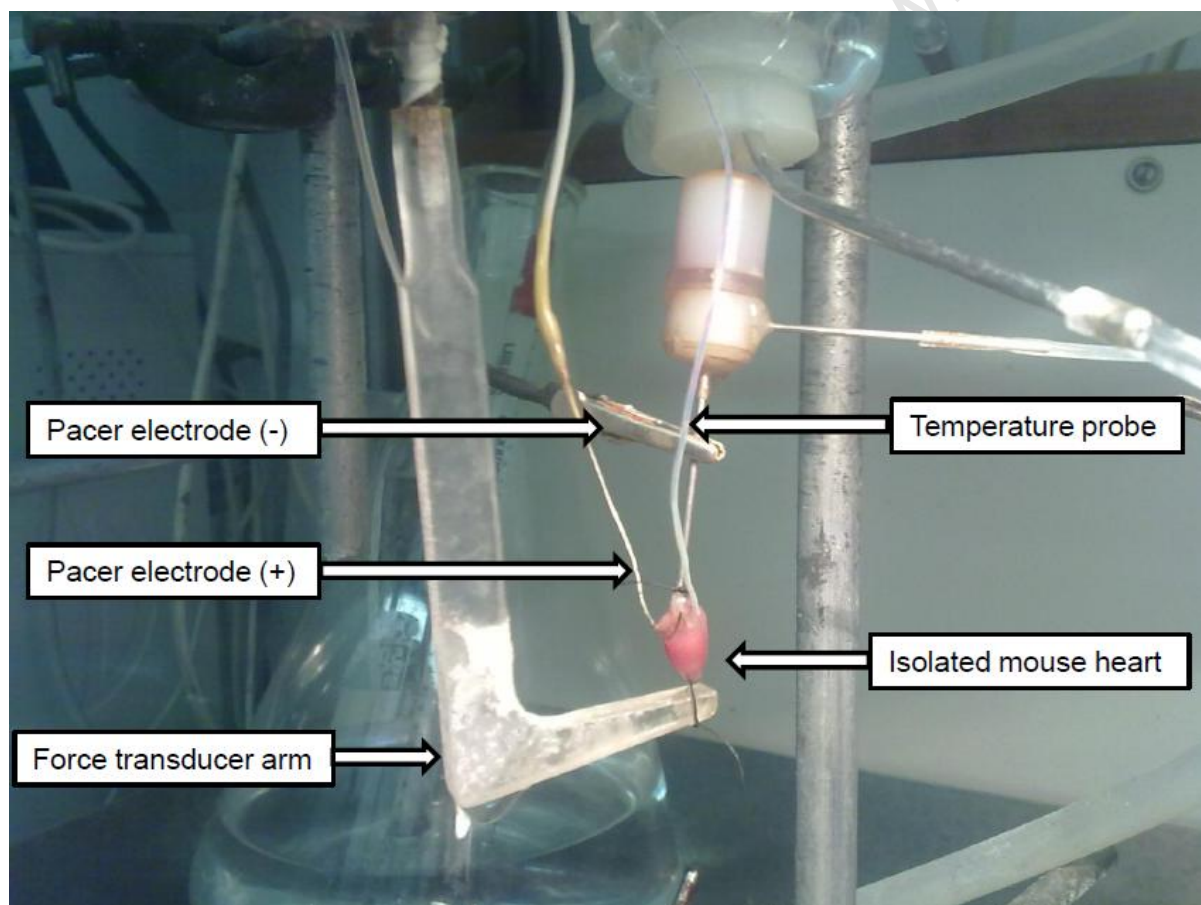


Fig 29: Mouse heart mounted on Langendorff apparatus. The heart is cannulated through its aorta in retrograde perfusion. Pacer electrodes are used to pace the heart to 560 bpm and tension applied against the heart is 2 g.

2.1. Infarct size assessment

At the end of the experiment, hearts were removed from the perfusion apparatus and stained with triphenyltetrazolium chloride (TTC, 1% in phosphate buffer, pH 7.4) for 1 minute at 37°C (described previously, (Sumeray et al., 2000)). Hearts were then frozen overnight and sectioned perpendicular to the apex, into 1 mm slices. The slices were tightly placed between two glass plates at a distance of 0.5 mm apart and digitally scanned. Infarct size was then calculated using computerized planimetry (Planimetry+, Boreal Software, Norway). The borders of inclusion and exclusion were analyzed blindly by another researcher and the same person to ensure consistency of results.

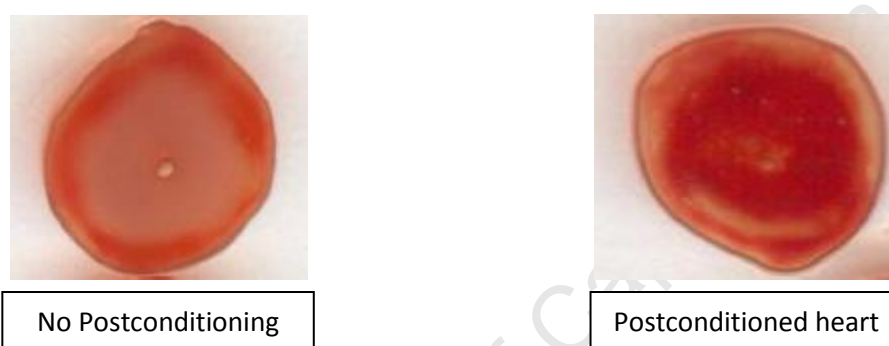


Fig 30. TTC staining for infarct size measurement. TTC reacts with the NADPH in live tissue and stains the cells with a brick red colour. The infarcted tissue is lacking in NADPH and remains pale. This distinction allows for the determination of infarct size by planimetry.

3.0. Nuclear and cytosolic protein extraction

This method of protein extraction (adapted from Williams and Ford, 2001, (Williams and Ford, 2001)) from mouse heart tissue involved the homogenization of tissue in the following lysis buffer:

<u>Solution A (30 ml)</u>	<u>μl</u>	<u>Final Concentration</u>
1 M Hepes, pH 7.9	600	20 mM
2 M MgCl ₂	37.5	2.5 mM
500 mM EDTA	6	100 μM
100 mM β-glycerophosphate	6000	20 mM

Triton X-100 (100%)	15	0.5 %
100 mM DTT	1500	500 μ M
100 mM NaVO ₄	30	100 μ M
EDTA-free protease inhibitor	1200	-
100 mM PMSF	300	1 mM
5 M NaCl	450	75 mM
dH ₂ O	19862	-

<u>Solution B (10 ml)</u>	<u>μl</u>	<u>Final Concentration</u>
1 M Hepes, pH 7.9	200	20 mM
2 M MgCl ₂	12.5	2.5 mM
500 mM EDTA	2	100 μ M
100 mM β -glycerophosphate	2000	20 mM
Triton X-100 (100%)	100	1.0 %
100 mM DTT	1500	500 μ M
100 mM NaVO ₄	10	100 μ M
EDTA-free protease inhibitor	400	-
100 mM PMSF	100	1 mM
5 M NaCl	150	75 mM
dH ₂ O	6526	-

Frozen hearts were wrapped in aluminium foil and pulverized under liquid nitrogen before transferred into 5 ml tubes containing 900 μ l of Solution A lysis buffer.

Samples were homogenized twice by Polytron at setting 4 for 5 seconds. The suspension was then centrifuged at 10000 g (12 000 rpm) for 5 minutes at 4°C. The supernatant containing the cytosolic fraction was collected and transferred into a fresh tube. The pelleted fraction was resuspended in 300-500 µl of Solution B and transferred to a clean micro-centrifuge tube. After centrifugation for 30 minutes at 10000 g (12 000 rpm) at 4°C, the supernatant containing the nuclear fraction was carefully removed and transferred to a clean tube.

3.1. Mitochondrial and cytosolic protein extraction

This method of mitochondrial and cytosolic protein extraction (adapted from Lewin et al., 2008, (Lewin et al., 2008)) from mouse heart tissue involved the homogenization of tissue in the following lysis buffer:

Lysis Buffer

100 mM Tris-HCl, pH 7.4

250 mM Sucrose

1 mM Dithiothreitol (DTT)

1 mM EDTA

Incubation buffer

250 mM Sucrose

25 mM Tris

8.5 mM KH₂PO₄

Low ionic strength sample buffer

400 µl 10 % Sodium dodecyl sulphate (SDS)

400 µl glycerol

40 µl mercaptoethanol

1.16 ml 5 mM Tris, pH 6.8

Bromophenol Blue

Frozen hearts were finely minced by scissors in 1 ml of lysis buffer and then transferred to a Dounce homogeniser. After homogenisation, the suspension was

centrifuged at 600 g for 5 minutes at 4°C. The supernatant was transferred to a fresh micro-centrifuge tube and centrifuged at 10 300 g (11 500 rpm) for 10 minutes. The supernatant is now the cytosolic fraction and the pellet, mitochondrial fraction. The pellet was resuspended in 40 µl incubation buffer. Proteins were quantitated and an equal volume low ionic strength sample buffer was added to each sample.

3.2. Lowry Protein assay

i) CTC reagent: Solution A - Na₂CO₃ in 20 g/100 ml double distilled water (ddH₂O)

Solution B - CuSO ₄ ·5H ₂ O	- 0.2 g	} 100 ml ddH ₂ O
K ₂ tartrate	- 0.4 g	

Add solution A to B with constant stirring to prevent precipitation.

ii) SDS

10 % SDS solution (w/v)

iii) NaOH

0.4 g/ 200 ml ddH₂O

Equal volumes of all three reagents were mixed immediately prior to use.

All samples were measured in duplicate. 5 µl of protein aliquot (nuclear, cytosolic, or mitochondrial sample) was added to 995 µl of sterile distilled water (1:200 dilution). 1 ml of CTC reagent was added to each tube and immediately mixed. After 10 minutes incubation at room temperature, 500 µl of Folin's Reagent (diluted 1:5 with ddH₂O, Merck, Germany) was added and mixed immediately - the half life of the reactive compound in the reagent is less than 8 seconds. Samples were incubated for an additional 30 minutes prior to optical density measurement at 750 nm on a Varian 130 dual beam spectrophotometer (Peterson, 1977).

Bovine Serum Albumin protein (Sigma, Germany) was used to prepare the standard curve and diluted to a concentration range from 10 – 200 µg/ml which was assayed in the same manner as the samples.

3.3. Western blot

Each well on the 10% (w/v) acrylamide/ bisacrylamide gel was loaded with 100 µg of nuclear, mitochondrial or cytosolic protein. Gels were run for 90-120 minutes at 120 Volts. 7 µl of PeqGold Protein marker IV (PEQLAB Ltd, UK) was loaded onto the gel and ran alongside the samples. Afterwards, gels were transferred overnight at 0.02 Amps (4°C) onto polyvinylidene fluoride (PVDF) membranes (Hybond, Amersham, UK). Proteins were fixed to the membrane by immersion in methanol for 5 minutes and air-dried. The success of protein transfer was verified by staining the PVDF membranes with Ponceau reagent (Sigma P7170, St Louis, MO). Membranes were placed between two acetate sheets and digitally scanned.

Ponceau stain was rinsed off the membranes with three 5 minute washes in Tris buffered saline with 0.1 % Tween (TBS-T). Membranes were blocked for 2 hours at room temperature with 5% fat-free milk powder made-up in TBS-T. Following three 5 minute cycles of washes with TBS-T, membranes were probed overnight at 4 °C with their respective primary antibody. Following another succession of three 5 minute washes with TBS-T, membranes were incubated with the appropriate secondary antibody.

3.4 Protein analysis

Isolated perfused mouse hearts samples were collected at reperfusion and snap-frozen in liquid nitrogen. Samples were stored at - 80 °C until protein extraction. Nuclear, cytosolic and mitochondrial proteins were extracted from hearts by homogenization in lysis buffer. Phosphorylated states of Akt, Erk1/2, STAT-3, GSK-3β as well as total levels of Akt, Erk1/2, STAT-3, GSK-3β, glyceraldehyde-3 phosphate kinase (GAPDH), β-actin and VDAC were analysed by western blot using antibodies from Cell Signaling Technologies. Equal loading was verified with GAPDH, β-actin or VDAC. These proteins are products of housekeeping genes that do not affect infarct size. Different proteins were selected to avoid conflicting molecular weight with the protein of interest and/or dependant on their localization. Levels of phosphorylated proteins were normalized, where appropriate, to their total protein levels in the sample samples and in the same conditions but on a separate membrane. Normalization represented the ratio of phosphorylated to total protein,

multiplied by 100. Relative densitometry was determined with the use of computerized software package (Image J, NIH, USA). A minimum of 4 hearts was used per group.

3.4.1 Primary and secondary antibodies

i) Total STAT-3

Primary antibody: STAT-3 antibody rabbit polyclonal IgG 1:4000 in 5 % (w/v) fat free milk TBS-T

Secondary antibody: Anti-rabbit 1:3000 in 5 % (w/v) fat free milk TBS-T

ii) Phosphorylated STAT-3

Primary antibody: phospho STAT-3 (Ser 727) antibody mouse monoclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-mouse 1:4000 in 2.5 % (w/v) fat free milk TBS-T

iii) Akt

Primary antibody: Akt antibody rabbit polyclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-rabbit 1:4000 in 2.5 % (w/v) fat free milk TBS-T

iv) Phosphorylated Akt

Primary antibody: Phospho-Akt (Ser473) antibody mouse polyclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-rabbit 1:3000 in 5 % (w/v) fat free milk TBS-T

v) Glycogen Synthase Kinase-3 Beta (GSK-3 β)

Primary antibody: GSK-3 β antibody rabbit monoclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-rabbit 1:4000 in TBS-T

vi) Phosphorylated Glycogen Synthase Kinase-3 Beta (GSK-3 β)

Primary antibody: Phospho-GSK-3 β (Ser9) antibody rabbit polyclonal IgG 1:3000 in TBS-T

Secondary antibody: Anti-rabbit 1:3000 in 5 % (w/v) fat free milk TBS-T

vii) p44/42 MAPK (Erk1/2)

Primary antibody: p44/42 (Erk1/2) antibody rabbit polyclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-rabbit 1:4000 in 2.5 % (w/v) fat free milk TBS-T

viii) Phospho- p44/42 MAPK (Erk1/2)

Primary antibody: Phospho-p44/42 (Erk1/2) (Thr202/Tyr204) antibody rabbit polyclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-rabbit 1:4000 in 2.5 % (w/v) fat free milk TBS-T

vii) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Primary antibody: GAPDH (FL-335) antibody rabbit polyclonal IgG 1:3000 in TBS-T

Secondary antibody: Anti-rabbit 1:4000 in 2.5 % (w/v) fat free milk TBS-T

vii) β actin

Primary antibody: β actin antibody goat polyclonal IgG 1:3000 in TBS-T

Secondary antibody: Anti-goat 1:4000 in 2.5 % (w/v) fat free milk TBS-T

viii) VDAC

Primary antibody: VDAC antibody goat polyclonal IgG 1:3000 in TBS-T

Secondary antibody: Anti-goat 1:4000 in 2.5 % (w/v) fat free milk TBS-T

All antibodies were acquired from Cell Signalling Technology, USA, except GAPDH (Santa Cruz Biotechnology, USA). Relative densitometry was determined with the use of computerized software package, Image J, National Institutes of Health, USA.

4.0. Preparation of High Density Lipoprotein (HDL)

HDL was isolated according to the protocol described by James et al., (James et al., 1989). The blood samples were collected and HDL was extracted at the University of Geneva Hospital, Switzerland. Blood was collected from healthy volunteers in EDTA coated tubes and spun for 10 minutes at 3500 rpm. The plasma content forms a yellow upper layer that was pooled together. Cumulative flotation ultracentrifugation was used to remove LDL and VLDL from the plasma where the volume of the plasma was adjusted against sodium bromide (NaBr) as follows:

$$\text{Plasma volume (ml)} \times 71.4 \text{ mg/ml} = \text{mg NaBr to be added,}$$

where 71.4 mg/ml is the pre calculated factor required to isolate VLDL and LDL from plasma.

Plasma containing NaBr was transferred to a 30 ml polyallomer centrifuge tube (Optiseal 361625, Beckman Coulter, USA) with aid of a syringe and spun for 21 hours at 20°C at 45 000 rpm. Afterwards, the tube was carefully removed from the centrifuge and placed in a custom made tube cutting apparatus (Fig 31). The VLDL and LDL was suspended at the top of the tube and separated by a clear middle layer from the HDL which resided in the bottom layer. The tube was cut at the distinction between the clear and VLDL/LDL layer. The remaining clear layer above the HDL fraction was carefully removed and discarded. The HDL fraction from all the tubes was pooled together into a measuring cylinder. The volume was noted and 204.1 mg/ml NaBr was added to it based HDL's density of 1.21 g/ml (calculated as earlier). The HDL was then decanted into another centrifuge tube and spun for 20 hours at 45 000 rpm at 20°C to further purify the HDL fraction. The HDL underwent delipidisation by passage through a gel filtration column in a 5M urea-phosphate buffered saline (PBS) solution. HDL purity was confirmed on a polyacrylamide gel stained with coomassie blue. The HDL was then passed through a pressurised concentrator apparatus before dialysis against PBS containing 1 mM EDTA to remove the urea.

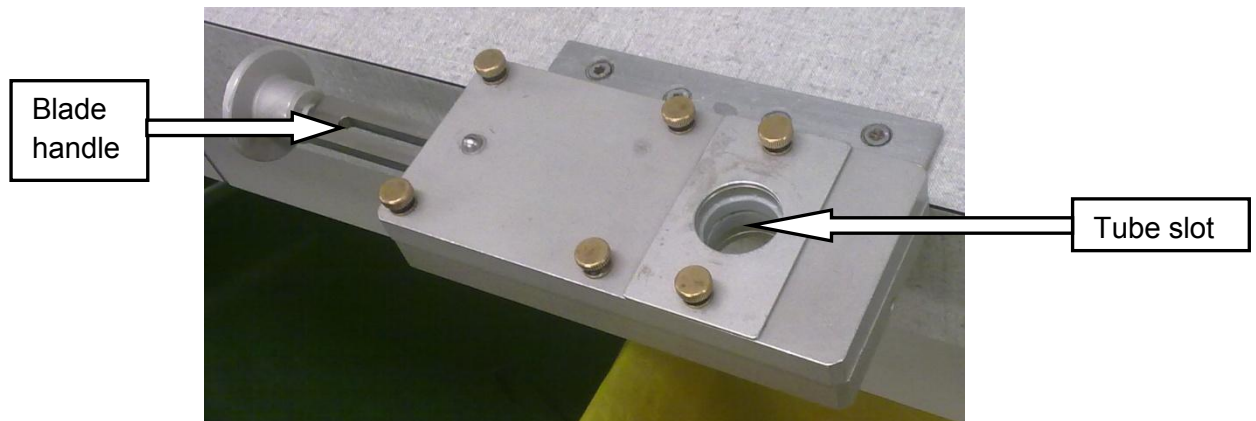


Fig 31. Custom built tube cutting apparatus. After ultracentrifugation, the polyallomer tube containing the HDL fraction was cut at the clear distinction layer between the HDL and VLDL/LDL layers.

University of Cape Town

D. ROLE OF STAT-3 IN ISCHAEMIC POSTCONDITIONING

1.0. Introduction

Despite the promising results of postconditioning, in experimental studies, its robustness across different animal models has yielded varying degrees of success. Reproducibility of the postconditioning effect has not always proved to be consistent. Previous studies have shown that some discrepancies may exist within the current rat and murine models as it proved less difficult to induce cardioprotection in the murine model across two laboratories (Kaljusto et al., 2006). In the seminal paper, Vinten-Johansson et al. were able to reduce significantly infarct size in the canine model by six alternating cycles of occlusion and reperfusion but translation of this powerful mechanism proved unsuccessful in the pig model (Zhao et al., 2003, Schwartz and Lagranha, 2006).

Postconditioning has successfully been achieved in the human heart by coronary angioplasty during acute myocardial infarction (Staat et al., 2005). This has further prompted the urgency to optimize the conditions required for this cardioprotective effect because the elucidation of its signaling mechanisms may provide the key to novel therapeutic drug development. STAT-3 (signal transducer and activator of transcription-3) is an important transcription factor that has been shown to be essential in mediating the cardioprotective effect for both ischaemic and pharmacological preconditioning (Smith et al., 2004, Lecour et al., 2005b). Cytosolic STAT-3 is phosphorylated by activation of the JAK-2 (Janus kinase-2) pathway and consequently forms a homodimer that translocates to the nucleus to initiate the transcription of pro-survival factors. Recently, phosphorylated levels of STAT-3 have also been detected in the mitochondria (Wegrzyn et al., 2009). There is increasing evidence to suggest that inhibition of the mitochondrial permeability pore (mPTP) prevents cell death. Glycogen synthase kinase 3-beta (GSK-3 β) has been reported as a common target for the convergence of protective signalling pathways and proximal to the mPTP (Halestrap et al., 2004, Gateau-Roesch et al., 2006, Halestrap et al., 2007). Phosphorylation of this kinase renders it inactive and it has been suggested that in this form it plays a role in the inhibition of the pore opening (Juhaszova et al., 2004).

In this study, we propose that age, genetic characterisation and the number of cycles are critical factors affecting postconditioning in an *in vitro* mouse heart model. In

addition, using cardiomyocyte specific STAT-3 deficient mice, we confirm the critical requirement of this transcription factor in IPostC and examine the possible involvement of GSK-3 β as a downstream target. A schematic of our proposed hypothesis follows in Figure 32.

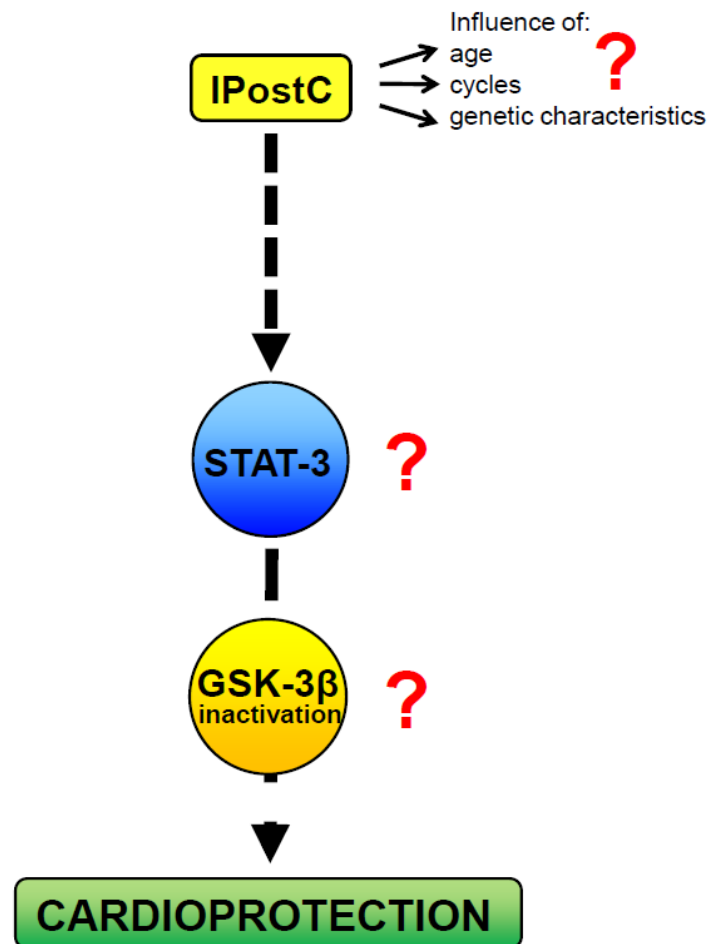


Fig 32. Schematic of proposed hypothesis. What factors favour ischaemic postconditioning? Are STAT-3 and/ or GSK-3 β involved? Abbreviations as described in the text.

2.0. Methods

The animal protocol was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, and was approved by the Faculty of Health Sciences Animal Ethics Committee (AEC), University of Cape Town (AEC reference number: 007/007). All protocols were carried out in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes for Health (NIH Publication No. 85 (23), revised 1996). Mice were bred and obtained from the University housed Animal Unit. Animals had access to food and water ad libitum. Cardiomyocyte-specific STAT-3-deficient mice (STAT-3^{-/-}) from C57Black6 background were created in our facilities by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre recombinase mice, as previously described (Smith et al., 2004).

2.1. Perfusion of isolated mouse hearts

We compared the postconditioning parameters in pure wildtype C57 Black 6 mice to the genetically modified rodents that only differed in respect to the presence of two *loxP* gene fragments inserted into the myosin light chain isoform-2 ventricle (MLC2V) expressing gene, which correspond to the wildtype used as a control for STAT-3 knockout studies. Wildtype male mice (C57BL/6) in the presence or absence of a floxed allele expression in the cardiac muscle and aged between 14-16 or 18-20 weeks were perfused on a Langendorff retrograde perfusion apparatus as previously described (see Section C). Following 20 min of stabilization, the hearts were exposed to 35 minutes of global ischaemia and 45 minutes of reperfusion, as described in figure 33. At the end of the experiment, hearts were stained with TTC to measure infarct size. Cardiomyocyte specific STAT-3 deficient mice were also perfused on the Langendorff apparatus to investigate the effect on postconditioning in the absence of this gene.

To examine STAT-3 and GSK-3 β levels, hearts were collected at 7 minutes of reperfusion following global ischaemia (Figure 33). Cytosolic and mitochondrial proteins were extracted and analysed by Western blot (refer to Section C).

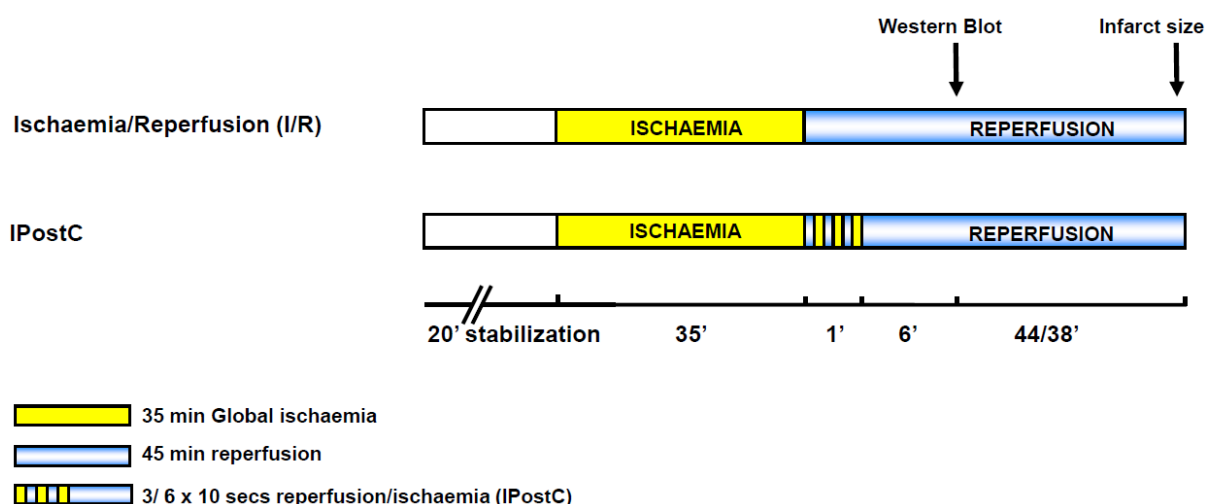


Fig 33. Schematic representation of experimental protocol. Mouse hearts subjected to 3 x 10 seconds of ischaemia/ reperfusion after 35 minutes global ischaemia were collected for Western blot analysis at the indicated time point in a separate experiment. *IPostC*, ischaemic postconditioning

2.2. Chemical and Pharmacological Agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemicals, Germany.

2.3. Statistical analysis

Data are expressed as mean values \pm S.E.M and were analysed by performing multiple group comparisons using one way ANOVA and followed by Student-Newman-Keuls post hoc test or T-Test (Graph Pad Instat). A value of $P < 0.05$ was considered to be statistically significant. A minimum of 6 hearts per group was used in this study.

3.0. Results

3.1. Postconditioning improves infarct size in young/ older wiltype non-floxed mice

To investigate the influence of age and number of cycles of postconditioning in wildtype mice, 14-16 or 18-20 weeks old mouse hearts were postconditioned with either 3 or 6 cycles of 10 seconds ischemia/reperfusion following the sustained ischemia (Figure 34). Ischaemic control hearts from young and older adult had a comparable infarct size of $48.6 \pm 1.5\%$ and $56.8 \pm 5.7\%$, respectively. Application of 3 cycles of postconditioning (IPostC-3) significantly reduced the ischaemic damage (infarct size of $14.2 \pm 0.9\%$ in young mice and $29.9 \pm 7.5\%$ in older mice). Similarly, postconditioning with 6 cycles of ischemia/ reperfusion (IPostC-6) also proved to be beneficial in both the young and adult wildtype mice ($16.2 \pm 2.6\%$ and $26.4 \pm 3.6\%$, respectively).

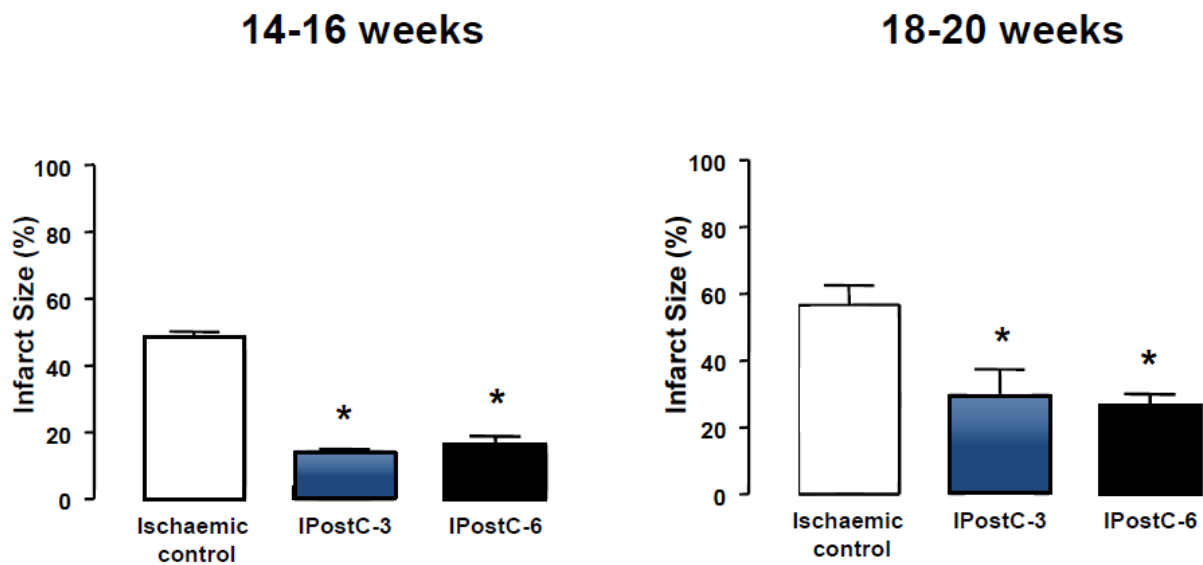


Fig 34. Postconditioning improves infarct size in younger/ older C57BL/6 non-floxed mice. ($n \geq 6$; $p < 0.05$ vs control group). *IPostC*, ischaemic postconditioning

3.2. Age and number of cycles of postconditioning affect wildtype floxed mice

As shown in Figure 35, at 14-16 weeks, infarct size which was attenuated by 3 cycles (26.2 ± 5.3%) but not by 6 cycles of postconditioning (40.9 ± 5.0%) compared to the ischaemic control (51.2 ± 4.2%). Postconditioning the older adult hearts with either 3 or 6 cycles produced an infarct size that did not differ appreciably from the ischaemic control (41.6 ± 1.7% for IPostC-3 and 38.4 ± 7.1% for IPostC-6 vs 37.9 ± 3.8% for ischaemic control). The main finding is that the protective effect of PostC-6 was lost in older group of floxed mice. However, the control infarct size in this group (37.9±3.8%) was smaller compared with the younger floxed group (51.2±4.2%) or the older non-floxed group (56.8±5.7%).

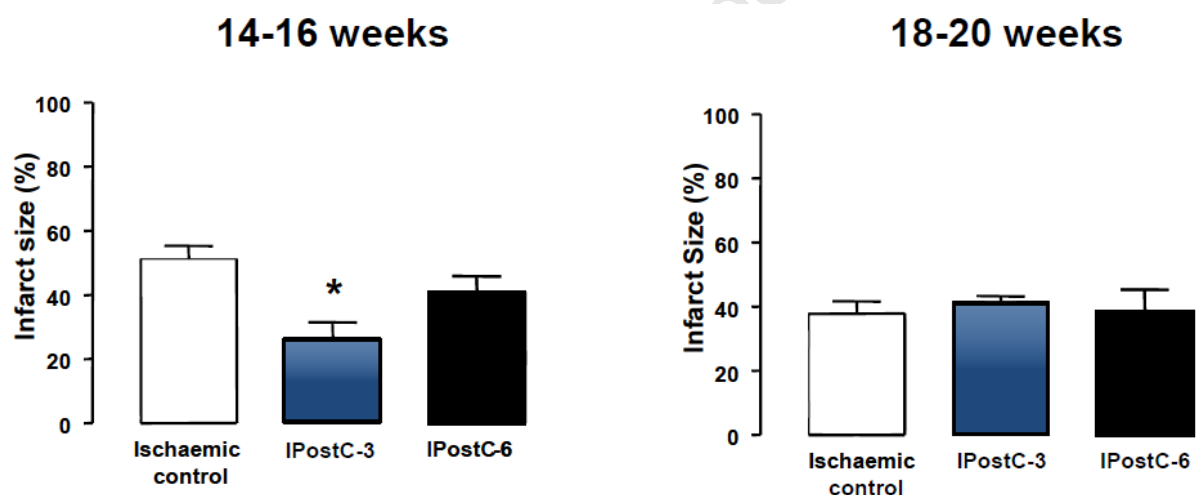


Fig. 35. Age and number of cycles influence the successful effect of postconditioning in floxed mice. (n ≥ 6; p < 0.05 vs control group). *IPostC*, ischaemic postconditioning

3.3. STAT-3 is required to confer cardioprotection in ischaemic postconditioning

Three cycles of postconditioning was applied to cardiomyocyte specific STAT-3 mice and its respective control, aged 14-16 weeks. As observed in the pure wildtype, the control wildtype mice were successfully postconditioned (51.2 ± 4.2 % for ischaemic control vs 26.2 ± 5.3 % for IPostC, $p < 0.05$, Figure 36). The protective effect was abolished in the absence of STAT-3 (49.5 ± 5.8 % for ischaemic control vs 49.6 ± 6.5 % for IPostC, $p = \text{ns}$).

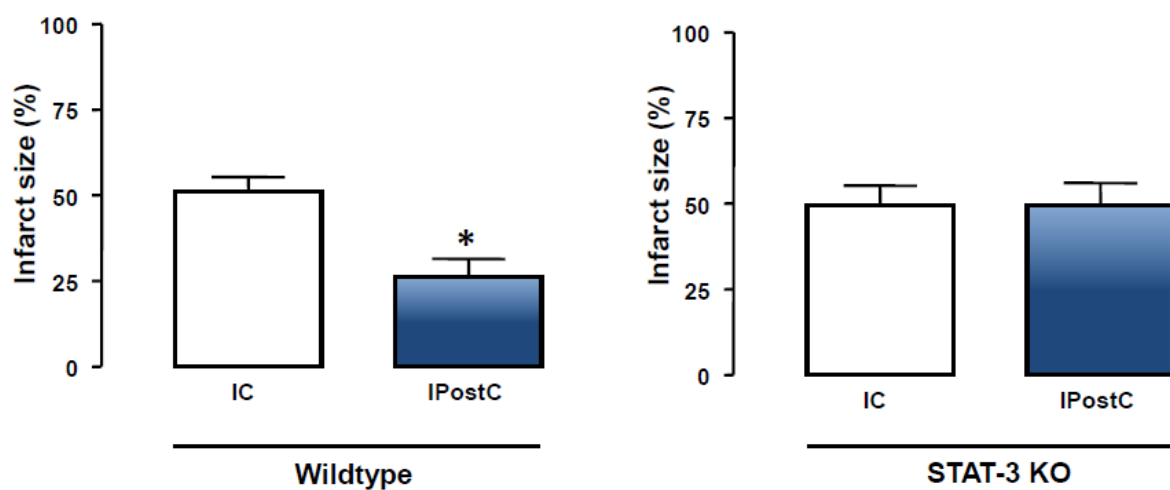


Fig. 36. The cardioprotective effect of ischaemic postconditioning is abolished in the cardiomyocyte specific STAT-3 knockout mice. ($n \geq 6$; $p < 0.05$ vs control group). IPostC, ischaemic postconditioning

4.0 Western Blots

4.1. Ischaemic Postconditioning increased the activation of mitochondrial STAT-3

To confirm the involvement of STAT-3 in IPostC, phosphorylated levels of STAT-3 were measured in the cytosol and mitochondria extracted from cardiomyocyte STAT-3 deficient hearts and their respective wildtype after 7 min of reperfusion. Compared to the wildtype ischaemic control, there was a marked reduction in phosphorylated STAT-3 in the cytosol following the IPostC stimulus (In arbitrary units (AU), 1.4 ± 0.3 AU for IC to 0.7 ± 0.3 AU for IPostC, $P < 0.05$, Figure 37). In contrast, phosphorylation of STAT-3 was significantly elevated in the mitochondrial fraction (0.4 ± 0.2 AU for IC to 0.8 ± 0.1 AU for IPostC, $P < 0.05$). This translocation effect was not observed in the cardiac deficient STAT-3^{-/-}. There was no significant difference between the ischaemic control and IPostc groups in the cytosolic (0.6 ± 0.3 AU for IC to 0.6 ± 0.2 AU for IPostC, $P = \text{ns}$) or mitochondrial fraction (0.4 ± 0.1 AU for IC to 0.4 ± 0.2 AU for IPostC, $P = \text{ns}$).

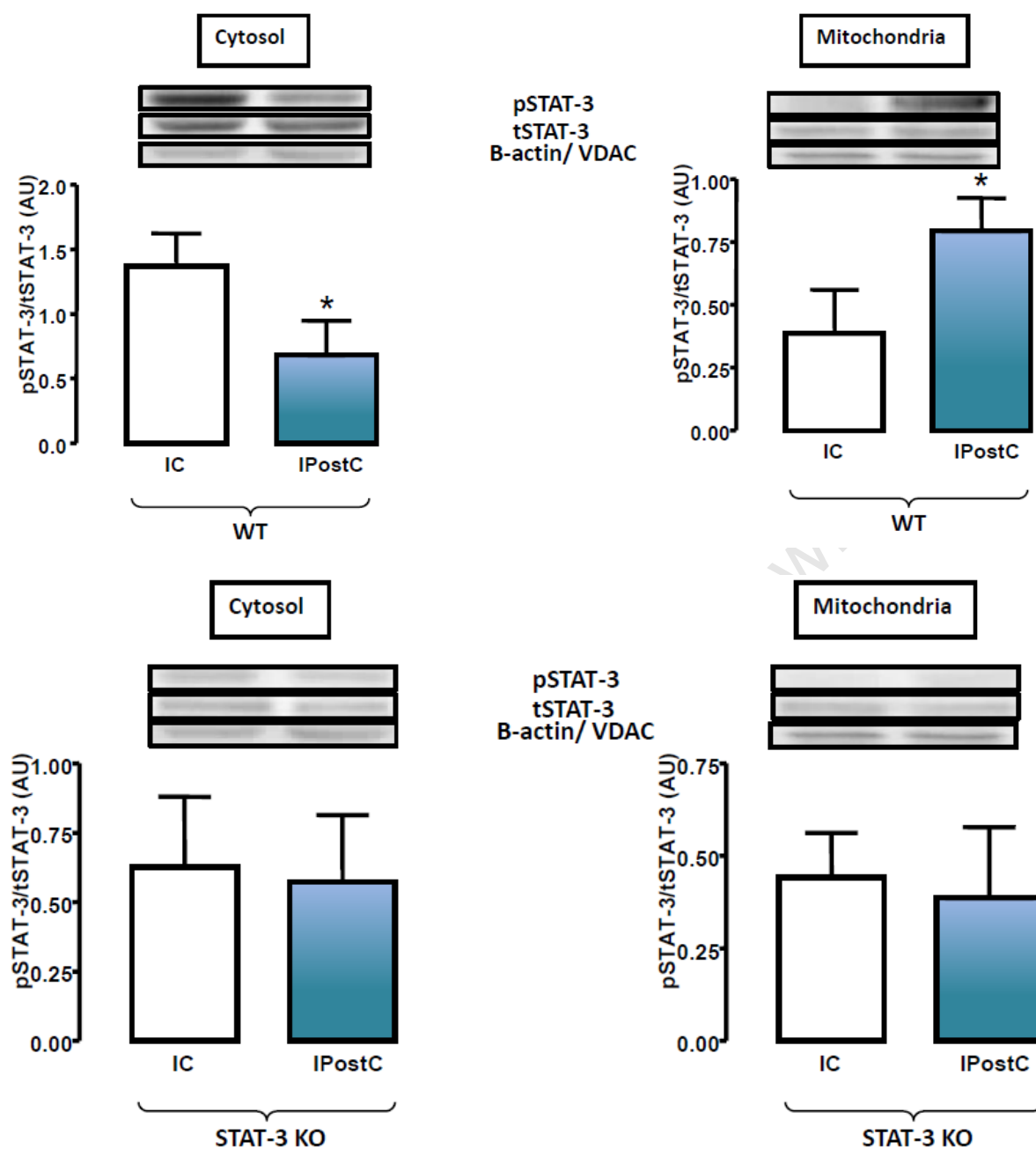


Fig 37. Western blot analysis of ischaemic postconditioning in STAT-3 deficient and their respective wildtype. IC, ischaemic control; IPOSTC, ischaemic postconditioning; STAT-3, Signal transducer and activator of transcription-3; WT, wildtype; KO, knockout

4.2. Glycogen synthase kinase 3 β levels are upregulated in ischaemic postconditioning

To explore whether GSK-3 β could be a possible downstream target of STAT-3, we examined levels of phosphorylated GSK-3 β in WT and STAT-3 KO mice subjected to IPostC. Phosphorylated levels of GSK-3 β were measured in the similar manner as STAT-3. In the cytosolic fraction of wildtype mouse hearts, there was a significant increase of phosphorylated GSK3 β levels in IPostC compared to the ischaemic control group (0.8 ± 0.1 AU for IC to 1.2 ± 0.1 AU for IPostC, $P < 0.05$). Similarly, phosphorylated GSK-3 β levels were elevated in the postconditioned mitochondrial fraction compared to the ischaemic control group (0.6 ± 0.0 AU for IC to 0.8 ± 0.1 AU for IPostC, $P < 0.05$). In the absence of STAT-3, GSK-3 β levels were unaffected in both the cytosolic and mitochondrial fraction (0.6 ± 0.2 AU for IC to 0.6 ± 0.1 AU for IPostC, $P = \text{ns}$; 0.6 ± 0.1 AU for IC to 0.7 ± 0.1 AU for IPostC, $P = \text{ns}$).

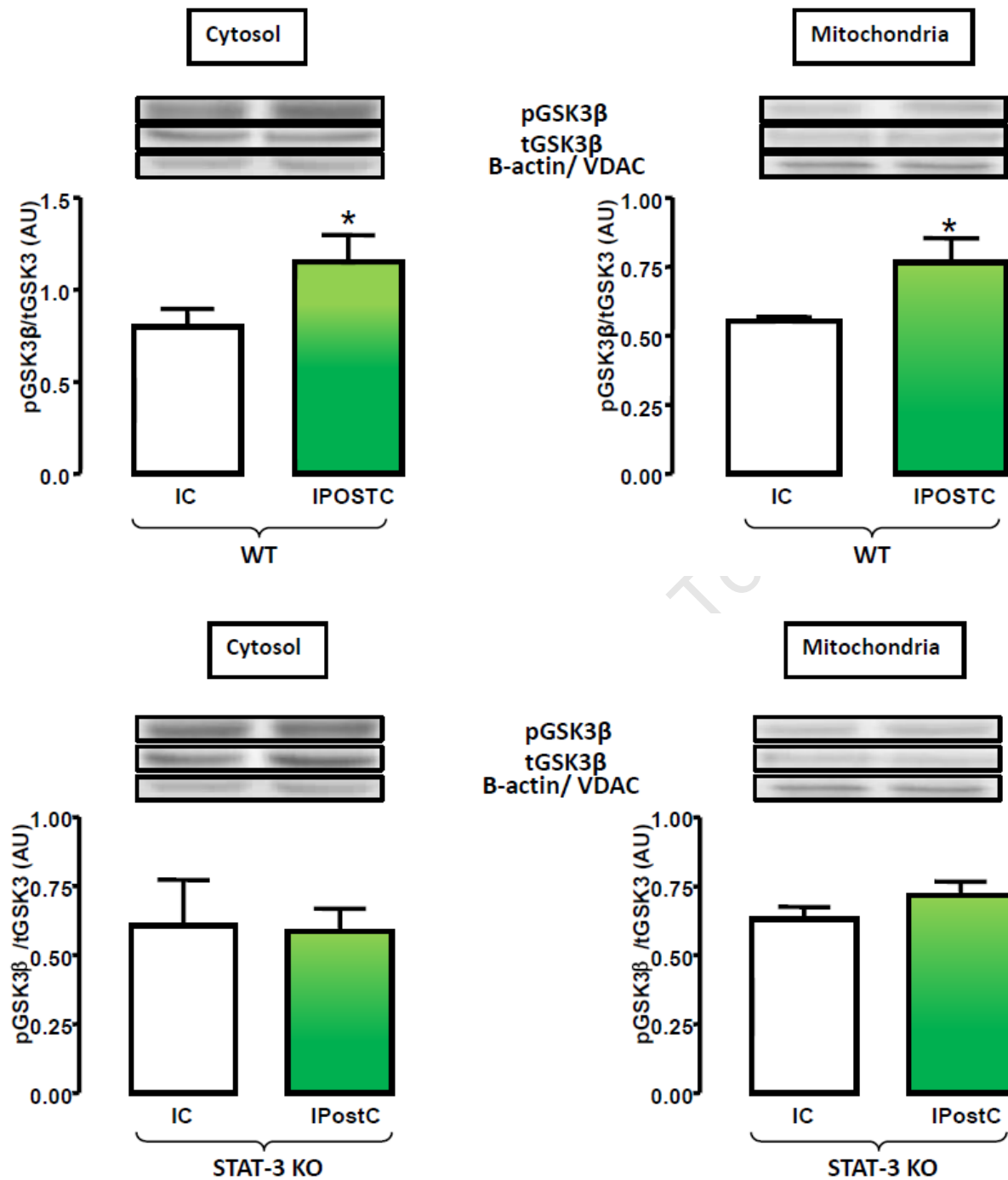


Fig 38. Phosphorylation of GSK-3β in wildtype and STAT-3 knockout mice. IC, ischaemic control; IPOSTC, ischaemic postconditioning; GSK-3β, Glycogen synthase kinase-3 beta; STAT-3, Signal transducer and activator of transcription-3; WT, wildtype; KO, knockout

5.0. Discussion

This study demonstrated that wildtype mice in the absence of a floxed gene are more prone to responding effectively to postconditioning. This was further evident with the older adults when a minor age difference of two weeks completely abolished the IPostC effect in the floxed mice. Additionally, the number of postconditioning cycles also played an important role in the wildtype strains that determined the extent of afforded cardioprotection. In addition, our data suggest that STAT-3 was essential in mediating the cardioprotective effect and involved in the regulation of phosphorylated GSK-3 β during IPostC.

5.1. Minor difference in age, number of cycles and genetic characterisation are critical parameters that can affect the outcome of ischaemic postconditioning

Postconditioning is a recently discovered phenomenon that can elicit profound protection if administered timeously following a myocardial infarction. It was first demonstrated successfully in an *in vivo* canine model of one hour left anterior descending (LAD) coronary artery occlusion using an algorithm of 30 seconds reperfusion and 30 seconds re-occlusion, repeated for 3 cycles and then continued reflow for the remaining 3 hours duration (Zhao et al., 2003). The resultant infarct size reduction was found to be strongly comparable to that of the phenomenon of ischaemic preconditioning (whereby small episodes of ischemia-reperfusion are performed prior to the major ischaemic insult (Murry et al., 1986). Interestingly, postconditioning across different animal models has not always met with similar levels of success. Kaljusto et al experienced difficulties when they conducted a study across two laboratories and while both groups were unable to postcondition the *in vitro* rat heart, only one group managed to successfully accomplished cardioprotection in the *in vivo* rat heart (Kaljusto et al., 2006). Discrepancies between the *in vitro* and *in vivo* model may be due to differences in antioxidant activity, neutrophils or other blood elements (Kin et al., 2004, Zhao et al., 2003, Kaljusto et al., 2006). Similarly, other studies have also had difficulties with postconditioning. Duplication of the postconditioning protocol as originally presented in the dog model failed to be cardioprotective in an *in vivo* pig study (Schwartz and Lagranha, 2006). Although mitochondrial volume differs substantially between the

dog and pig heart, it is unlikely to affect postconditioning. All reported studies have been conducted in animals housed in an animal facility.

Postconditioning reduced lethal ventricular arrhythmias in an *in vivo* rat model but failed to reduce infarct size (Dow and Kloner, 2007). All these discrepancies suggest that many critical factors are not controlled yet and need to be taken into consideration.

A successful postconditioning outcome is also influenced by the number and length of cycles of ischemia and reperfusion that follow the prolonged ischemia. Yang et al. showed that alteration of the postconditioning cycles afforded protection in the isolated rabbit heart model (Yang et al., 2005). Similarly, our experiments demonstrated that only postconditioning with 3 cycles was successful in reducing infarct size in the floxed mice at 14-16 weeks whereas postconditioning the hearts in non-floxed mice appeared more robust. An insertion of 2 loxP gene fragments into the MLC2V targeted gene is usually thought to be silenced but may influence the successful effect of postconditioning. STAT-3 is a transcription factor that is essential in mediating the cardioprotective effect of ischaemic postconditioning (Boengler et al., 2008, Lacerda et al., 2009). However, abnormal alterations in STAT-3 levels have shown to be detrimental to the heart. The lack of cardiac STAT-3 lead to dilated cardiomyopathy and overstimulation of STAT-3 caused inflammation, adverse remodeling and heart failure (Hilfiker-Kleiner et al., 2004, Hilfiker-Kleiner et al., 2010). Although never described in the literature, STAT-3 may have a critical role in the development process of the heart, which the insertion of lox P may interfere with at 16-18 weeks. An effect of lox P has previously been reported with hyperandrogenization in male mice containing a floxed allele of the androgen receptor gene (MacLean et al., 2008).

An age difference study conducted by Fenton et al. showed that the absence of postconditioning-induced protection in rats at 22 months (Fenton et al., 2000). In our study, we were surprised to report that a minor age difference in floxed mice from 14-16 weeks to 18-20 weeks can negatively affect the outcome of postconditioning. This was in contrast with the hearts from genetically unmodified

wildtype mice which were still able to be postconditioned at 18-20 weeks. A minor gene modification to allow for the creation of genetic knockout mice should not be expected to interfere with the initiation of cardioprotection. Our data reinforce the concept that studies employing the use of genetically modified animals should strongly consider the littermate controls instead of simply using wildtype mice in their control group, especially with regard to studies related to cardioprotection. Recently, it was demonstrated that a modification to a more robust postconditioning protocol (extension of the number of cycles) was required to induce protection in an *in vitro* aged (> 13 months) mouse model (Bouhidel et al., 2008).

Other critical factors influencing postconditioning also need to be considered. The beneficial effects of postconditioning are limited to coronary occlusions of less than 45 min in the *in vivo* rat model (Tang et al., 2006). Also, the maintenance of acidosis by postconditioning during the first minutes of reperfusion is critical to inhibit opening of the *mPTP* (Cohen et al., 2007). Temperature is another important factor to be considered. The successful outcome of postconditioning in the *in vitro* rat heart was recently shown to be highly dependent upon the maintenance of temperature at 37°C as the slightest fluctuations abrogated its protective effect (van Vuuren et al., 2008). Alteration in body temperature may create an unfavourable environment for essential enzymatic reactions. It is also possible that divergent pathways may exist not only from preconditioning but also between animal models of postconditioning. Activation of ERK was exhibited in the absence of PI3K-Akt in the postconditioned rabbit model (Darling et al., 2005). This is in contrast to PI3K-Akt involvement in the pre- and postconditioned *in vitro* rat heart model (Tsang et al., 2004). It was also possible to postcondition the pig model using a modified protocol that was independent of these kinases, previously thought essential for protection (Skyschally et al., 2009).

Obesity is a common cause of cardiovascular disease. In a recent study, postconditioning failed to induce cardioprotection in leptin-deficient (*ob/ob*) mice (Bouhidel et al., 2008). In addition, there are a number of risk factors such as diabetes, hyperlipidemia, insulin resistance, hypertension and heart failure that may also influence the success of postconditioning.

5.2. STAT-3 is required for ischaemic postconditioning

STAT-3 has been previously demonstrated as a critical requirement in cardioprotective studies (Smith et al., 2004). At the time of this study, its role in IPostC had yet to be determined and verified in a cardiac specific model. Suleman et al. abolished the infarct sparing effect ischaemic postconditioning with JAK2 inhibitor, AG490 in the isolated rat heart model (Suleman, 2006). Decreased cytosolic levels of phosphorylated STAT-3 following IPostC, was observed by western analysis, suggesting activation and nuclear translocation of STAT-3. Schulz and colleagues also confirmed the essential role for STAT-3 in IPostC in an *in vivo* cardiac specific deficient STAT-3 mouse model (Boengler et al., 2008). Similarly, an interaction between the JAK/STAT-3 pathway and PI3-kinase/ Akt pathway to explain IPostC's beneficial effects is suggested (Goodman et al., 2008). Recently, we demonstrated the activation of the prosurvival SAFE pathway in IPostC (Lacerda et al., 2009). TNF α was shown to mediate the protective signal via its TNF receptor 2 and STAT-3. IPostC failed to reduce infarct size in TNF $^{-/-}$ and TNFR2 $^{-/-}$ mice. In this study, mouse hearts were also pharmacologically postconditioned with TNF α which was dependant on the activation of STAT-3 but independent of the RISK pathway, which involves the PI3-K/ Akt and ERK1/2 kinases.

Inactivation of GSK-3 β by phosphorylation has been established as a downstream target in cardioprotection and implicated in mitochondrial permeability pore inhibition (Juhaszova et al., 2009, Gross et al., 2006). However, its mechanism of action is unclear. We were interested in whether or not GSK-3 β may be regulated by STAT-3 in IPostC. Our studies showed that phosphorylated GSK-3 β was elevated in both cytosolic and mitochondrial fractions obtained from the wildtype hearts following IPostC. Our findings are consistent with those of Nishihara et al. who also demonstrated a mitochondrial translocation of GSK-3 β following an ischaemic preconditioning stimulus (Nishihara et al., 2007). It is suggested that GSK-3 β translocation may employ Translocase of the Outer mitochondrial Membrane 20 (TOM20) (Rodriguez-Sinovas et al., 2006). In our experiments, GSK-3 β levels were unaffected in STAT-3 $^{-/-}$ hearts with IPostC treatment, suggesting that STAT-3 may be involved in the regulation of this kinase in IPostC. However, this does not prove that GSK-3 β is essential to IPostC induced protection and its involvement has been questioned. A study conducted by Marber and colleagues showed that they were still

able to successfully postcondition hearts from homozygous GSK-3 double knockin mice (GSK-3 α and GSK-3 β isoforms were inhibited) (Nishino et al., 2008). In contrast, Juhaszova et al. demonstrated that GSK-3 β was essential for protection in their cardiomyocyte model (Juhaszova et al., 2004). Genetically modified mice expressing constitutively active GSK-3 β that cannot be inactivated by phosphorylation of its serine 9 residue, failed to be postconditioned and GSK-3 β inactivation was suggested to exert its cardioprotective effects via inhibition of the mPTP (Juhaszova et al., 2004). It may be possible that under certain conditions, GSK-3 β is regulated by divergent signalling mechanisms.

5.3. Conclusion

Postconditioning represents a means to harness the myocardium's endogenous capacity to resist damage. The aged heart has an increased susceptibility to damage and therefore it is imperative to unravel the complexity of its signaling mechanisms so that novel therapeutic agents may be developed to elicit postconditioning. Our data strongly suggests that the genotypic characteristics of the mouse, the age and the number of postconditioning cycles are critical factors to be considered in ischaemic postconditioning. Different animal models may require different postconditioning protocols. Additional factors such as humidity and stress also require investigation. It might be possible that the discrepancies observed in the floxed mice may be due to the dysregulation of gene programs that attenuate the postconditioning effect. These factors should be taken into consideration for future experimental research or clinical applications of this protective phenomenon. STAT-3 has also been demonstrated as an essential factor in ischaemic postconditioning. Since it forms part of the prosurvival SAFE pathway, it would be interesting to further explore its role in cardioprotection as it may represent a novel therapeutic target against reperfusion injury.

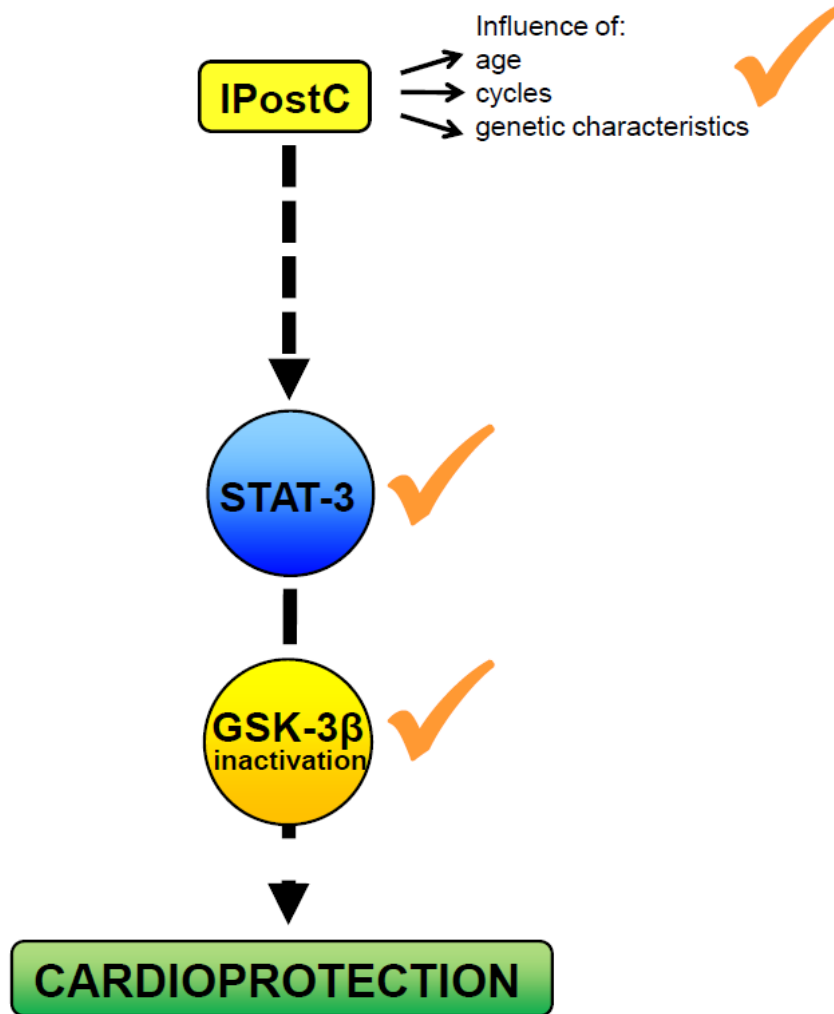


Fig 39. Summary of our data. Age, postconditioning cycles and genetic characteristics are critical to the successful outcome of ischaemic postconditioning. STAT-3 is an essential to mediating cardioprotection but GSK-3 β 's role is questionable. *IPostC*, ischaemic postconditioning; *STAT-3*, Signal Transducer and Activator of Transcription-3; GSK-3 β , glycogen synthase kinase 3 beta

E. CLASSICAL PHARMACOLOGICAL AGENTS USED AS POSTCONDITIONING MIMETICS: ROLE OF STAT-3

Part of the experiments in this section have been performed in conjunction with Dr Lydia Lacerda at the University of Cape Town

1.0. Introduction

Ischaemic postconditioning represents a promising clinical intervention to reduce myocardial infarction to the ischaemic heart. However, its clinical application, by inflation/ deflation of the balloon or with the pressure cuff may not always be practical, as it needs to be performed at a critical time when every minute counts to save the life of the patient. Pharmacological postconditioning with the administration of a drug would certainly be more practical. For this, a greater understanding of the mechanisms involved in the postconditioning phenomenon is required. Adenosine, bradykinin and opioids are three compounds released during ischaemic preconditioning but it is unknown whether these drugs may postcondition the heart.

In the previous section of this thesis, we demonstrated that ischaemic postconditioning can mediate cardioprotection via the activation of the SAFE pathway involving TNF α and STAT-3 (Lacerda et al., 2009). But the role of the SAFE pathway in adenosine, bradykinin and opioid induced cardioprotection has never been explored.

Therefore, the aim of this study was to explore the benefit of pharmacological postconditioning with adenosine, bradykinin and opioids compared to ischaemic postconditioning and to investigate the role of the SAFE pathway as the cardioprotective effect.

To explore this aim, TNF α knockout, cardiomyocyte specific STAT-3 deficient mice (STAT-3^{-/-}) and their respective wildtypes will be subjected to an ischaemia/ reperfusion insult on the isolated mouse heart model. Ischaemic or pharmacological postconditioning (with adenosine, bradykinin and opioids) will be performed at the onset of reperfusion and infarct size will be used as an end point. Since STAT-3 has been recently identified in the mitochondria, an organelle considered as the putative end target for postconditioning, we used the western blot technique to evaluate the possible activation of STAT-3 in the mitochondria following a pharmacological stimulus (Wegrzyn et al., 2009, Heusch et al., 2010). A schematic of our proposed hypothesis follows in figure 40.

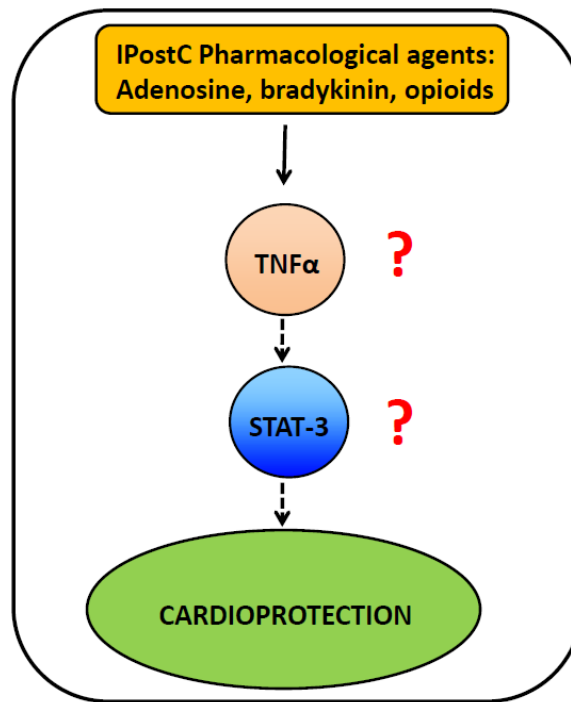


Fig 40. Schematic of proposed hypothesis. Pharmacological postconditioning with adenosine, bradykinin and opioids induce cardioprotection via activation of TNF α and the subsequent phosphorylation and mitochondrial translocation of STAT-3. TNF α , Tumour Necrosis Factor- α ; JAK/STAT-3, Janus Activated Kinase/ Signal Transducer and Activator of Transcription-3

2.0. Methods

All animal studies performed in this chapter complied with the recommendations laid down in the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85 (23), revised 1996) and were approved by the the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town (AEC reference number: 006/021 and 010/033). Six mice were used per experimental group. Cardiomyocyte-specific STAT-3-deficient mice (STAT-3^{-/-}) from C57Black6 background were created in our facilities by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre recombinase mice, as previously described (Smith et al., 2004). Homozygous TNF α -deficient mice were also used. Mice used in this study were 12-14 weeks of age.

2.1 Pharmacological postconditioning with classic cardioprotective agents

Adenosine (100 μ M), bradykinin (100 nM), and delta opioid agonist, D-Ala 2', D-Leu 5'- enkephalin acetate salt (DADLE, 100 nM) were given at the onset of reperfusion as postconditioning mimetics to isolated mouse hearts from TNF^{-/-} and STAT-3^{-/-} mice and their respective wildtypes.

2.2.1 Perfusion of mouse hearts

Hearts from adult male TNF α ^{-/-} and STAT-3^{-/-} mice and their respective wildtypes were isolated and perfused on the Langendorff retrograde apparatus as described in Section C. An IPostC protocol of 6 x 10 seconds alternating cycles of ischaemia/reperfusion (I/R) was selected because TNF α ^{-/-} mouse hearts could not be protected with 3 x 10 seconds alternating cycles of I/R at 12-14 weeks of age. Adenosine, bradykinin and DADLE were given to the hearts at the onset of reperfusion following the extended ischaemic insult via 6 x 10 seconds alternating cycles of reperfusion and reperfusion together with each of the pharmacological agents in turn as outlined in figure 41.

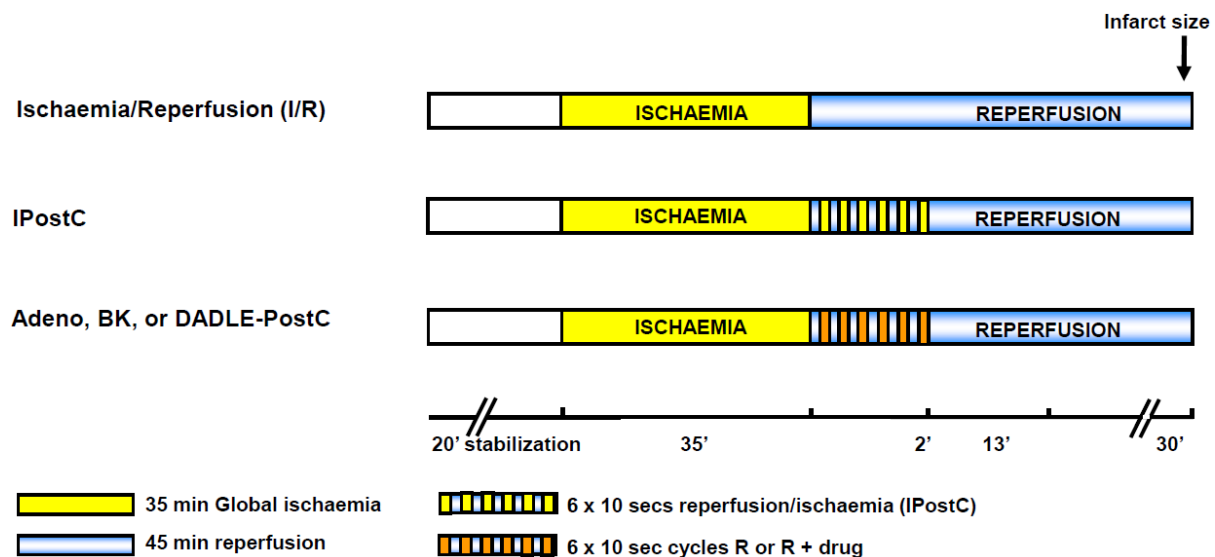


Fig 41. Schematic of the Langendorff experimental protocol. Isolated mouse hearts were perfused for 20 minutes followed by 35 minutes global ischaemia and a 45 minute reperfusion period. Ischaemic postconditioning (IPostC) was initiated at the onset of reperfusion with 6 x 10 seconds of alternating of ischaemia/ reperfusion. In a separate group, pharmacological postconditioning with 100 μ M adenosine, 100 nM Bradykinin or 100 nM DADLE was initiated with 6 x 10 secs of intermittent reperfusion.

2.2.2 Infarct size assessment

At the end of the experiment, hearts were stained with triphenyltetrazolium chloride to measure infarct size, as described previously (Smith et al., 2002). Hearts were then frozen overnight and sectioned into 1.5 mm slices. The slices were tightly placed between two glass plates at a distance of 0.5 mm apart and digitally scanned. A minimum of 6 hearts per group was used in this study. Infarct size was calculated using computerized planimetry (Planimetry+, Boreal Software, Norway).

2.3. Western Blot analysis

Control or postconditioned hearts with adenosine, bradykinin, or DADLE, were subjected to 20 min stabilization, 35 min global ischaemia followed by 15 min reperfusion. At this point, hearts were snap frozen in liquid nitrogen and stored at -80°C, until protein extraction could be performed. Cytosolic and mitochondrial extractions were performed as previously described in Section C. Phosphorylated and total levels of STAT-3 (Phospho-STAT-3, ser 737), were analysed by sodium

dodecyl sulphate polyacrylamide gel immunoelectrophoresis (SDS-PAGE). Equal loading was verified by measuring levels of β -actin (cytosol) or VDAC (mitochondria). Relative Densitometry analysis was carried out using a computerized software package (Image J analysis and processing in Java). A minimum of six hearts per group were used.

2.4. Chemicals and pharmacological agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich chemicals, Germany. Western blot antibodies were obtained from Cell Signaling Technologies, USA and Santa Cruz Biotechnology, Inc, USA.

2.5. Statistical analysis

Data are expressed as mean values \pm S.E.M and were analysed by performing multiple group comparisons using one way ANOVA and followed by Student-Newman-Keuls post hoc test or T-test (Graph Pad Instat). A value of $P < 0.05$ was considered to be statistically significant.

3.0. Results

3.1. Pharmacological postconditioning with classic cardioprotective agents in the presence or absence of TNF

Ischaemic postconditioning significantly improved infarct size in the TNF-WT mice compared to the ischaemic control group (IC) ($17.1 \pm 1.2\%$ for IPostC vs $46.0 \pm 0.8\%$ for IC, $p < 0.05$, Figure 42). Similarly, bradykinin, DADLE and adenosine attenuated the infarct size in TNF-WT hearts ($19.0 \pm 1.1\%$ for bradykinin postconditioning (Bk-PostC), $23.8 \pm 1.3\%$ for DADLE postconditioning (D-PostC), and $14.7 \pm 1.3\%$ for adenosine postconditioning (Adeno-PostC) vs IC, $p < 0.05$). In contrast, this protective effect was abolished in IPostC, Bk-PostC and D-PostC hearts in the absence of the TNF α gene ($47.8 \pm 1.3\%$ for IPostC, $47.1 \pm 1.4\%$ for Bk-PostC, and $48.9 \pm 1.2\%$ for D-PostC vs $47.1 \pm 1.2\%$ for IC, $p = \text{ns}$). Surprisingly, adenosine induced a cardioprotective effect in TNF α -deficient mouse hearts against ischaemia reperfusion injury ($19.2 \pm 1.3\%$ for Adeno-PostC vs IC, $p < 0.05$).

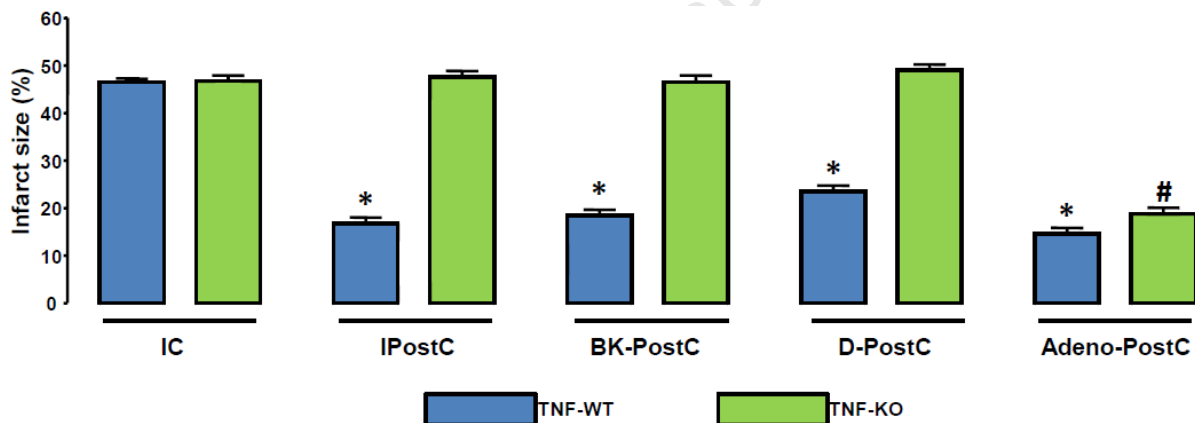


Fig 42. Classic cardioprotective agents fail to protect hearts in TNF α ^{-/-} mice except for adenosine. * $p < 0.05$ vs IC (TNF-WT), # $p < 0.05$ vs IC (TNF α ^{-/-}), $n=6$. IC, ischaemic control; Bk-PostC, bradykinin postconditioning; D-PostC, DADLE postconditioning; Adeno-PostC, adenosine postconditioning

3.2. STAT-3 is essential for pharmacological postconditioning with bradykinin, DADLE and adenosine

In STAT-3-WT mice, ischaemic postconditioning reduced infarct size from $46.0 \pm 2.8\%$ in the ischaemic control group to $23.1 \pm 4.5\%$ for IPostC (Figure 43, $p < 0.05$). Similarly, Bk-PostC, D-PostC and Adeno-PostC also protected STAT-3-WT hearts by reducing infarct size from $39.9 \pm 2.4\%$ in the ischaemic control group to $20.1 \pm 4.1\%$, $18.2 \pm 1.7\%$, and $21.9 \pm 2.6\%$, respectively ($P < 0.05$ vs IC). None of the postconditioning protocols were effective in reducing infarct size in the STAT-3 deficient mice ($49.8 \pm 5.4\%$ for IPostC, $41.3 \pm 2.7\%$ for Bk-PostC, and $39.2 \pm 1.9\%$ for D-PostC, and $37.2 \pm 4.6\%$ for Adeno-PostC vs $39 \pm 2.4\%$ for IC, $p = \text{ns}$).

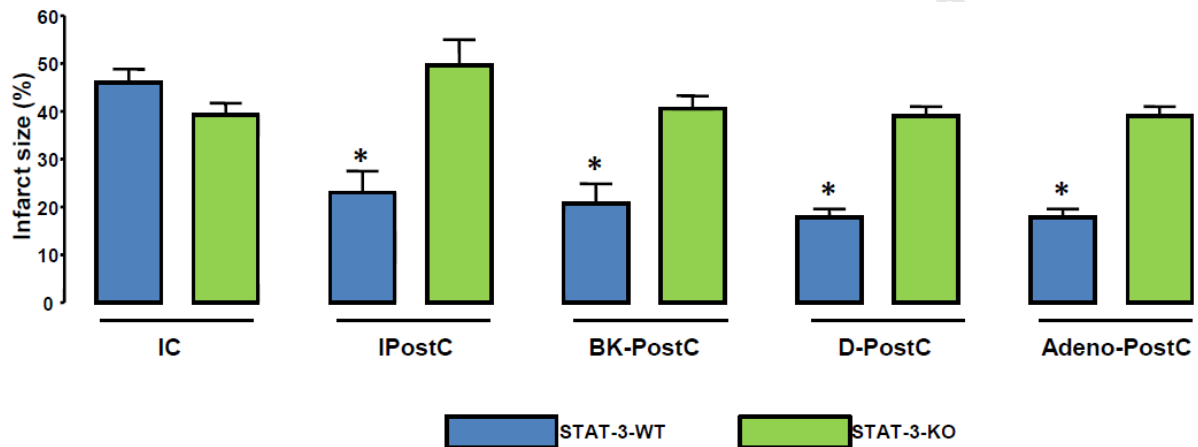


Fig 43. The beneficial effect of postconditioning with bradykinin, DADLE and adenosine is cardioprotective in STAT-3-WT but lost in STAT-3^{-/-} mice. * $p < 0.05$ vs IC (TNF-WT), $n=6$. IC, ischaemic control; *Bk-PostC*, bradykinin postconditioning; *D-PostC*, DADLE postconditioning; *Adeno-PostC*, adenosine postconditioning

3.3. Western blot analysis

The mitochondrial and cytosol activation of STAT-3 was investigated after pharmacological postconditioning in TNF-WT and TNF $\alpha^{-/-}$ mice (Figure 44). In the mitochondria isolated from TNF-WT hearts, we observed a significant increase in phosphorylated STAT-3 after postconditioning with bradykinin and DADLE (1.6 ± 0.2 AU for Bk-PostC, and 1.8 ± 0.2 AU for D-PostC vs 0.8 ± 0.2 AU for IC, $p < 0.05$) but not with adenosine (1.2 ± 0.1 AU for Adeno-PostC vs IC, $p = \text{ns}$). In contrast, there was no significant change in STAT-3 phosphorylation following treatment of the postconditioning mimetics in TNF $\alpha^{-/-}$ mitochondria (1.3 ± 0.3 AU for Bk-PostC, 1.7 ± 0.3 AU for D-PostC and 1.6 ± 0.4 AU for Adeno-PostC vs 1.5 ± 0.3 AU for IC, $p = \text{ns}$). In the WT cytosolic fraction, phosphorylated STAT-3 tend to decrease following the treatment with all postconditioning mimetics but only D-PostC showed a significant decrease in STAT-3 phosphorylation (0.7 ± 0.1 AU for D-PostC vs 1.3 ± 0.2 AU for IC, $p < 0.05$). In the absence of TNF α (as in TNF $\alpha^{-/-}$), there was no significant increase in cytosolic phosphorylation observed in any of the postconditioned groups ($p = \text{ns}$).

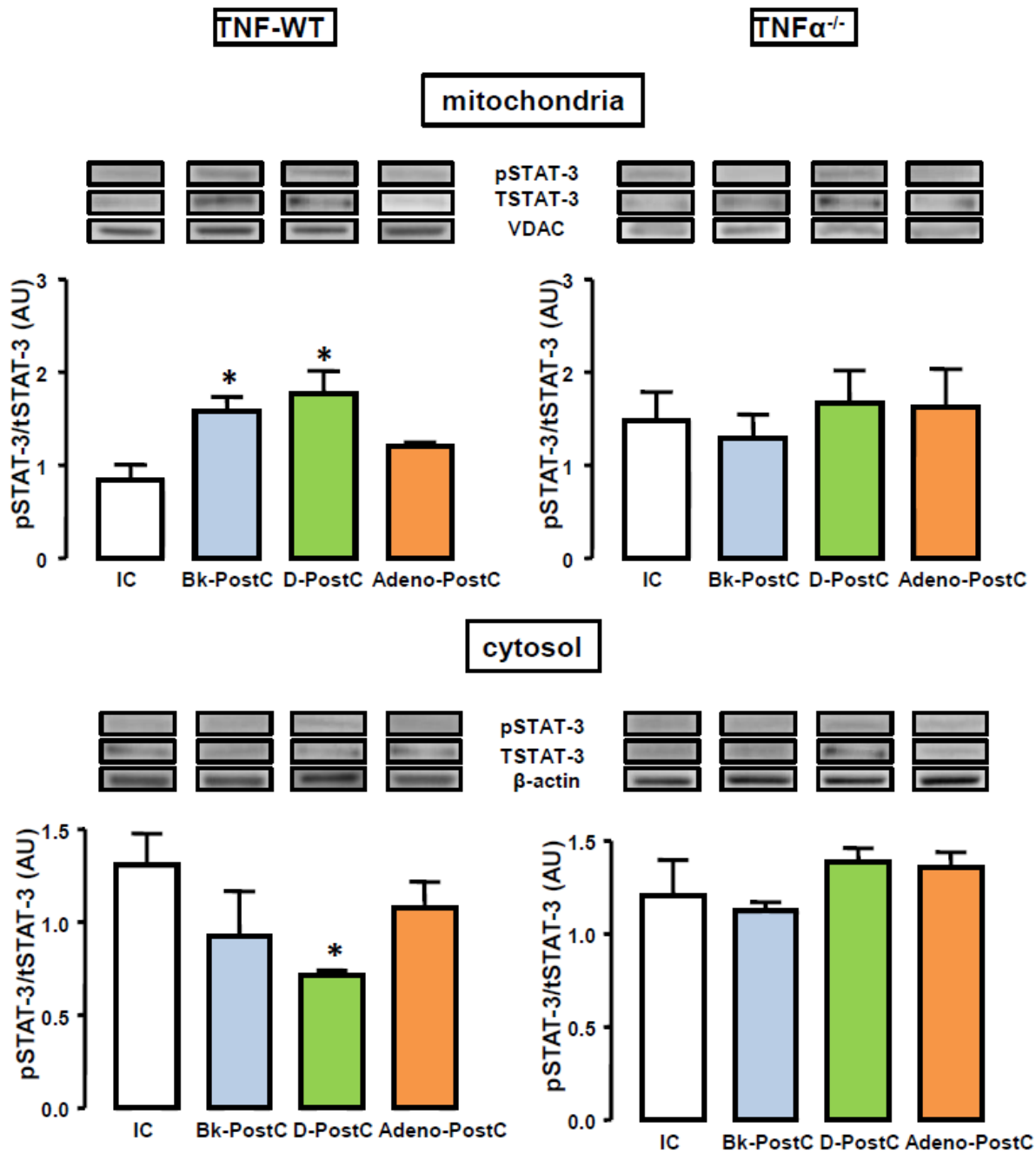


Fig 44. The mitochondrial translocation of phosphorylation STAT-3 following pharmacological postconditioning with bradykinin, DADLE and adenosine. *p < 0.05 vs IC, n=4. IC, ischaemic control; *Bk-PostC*, bradykinin postconditioning; *D-PostC*, DADLE postconditioning; *Adeno-PostC*, adenosine postconditioning

4.0. Discussion

In this study, we investigated the postconditioning potential of classic pharmacological agents, namely, adenosine, bradykinin and opioids. Pharmacological postconditioning with these agents conferred cardioprotection in WT isolated mouse hearts subjected to an ischaemia/ reperfusion insult to a similar extent to ischaemic postconditioning. Interestingly, we found that bradykinin and opioids conferred cardioprotection via activation of the SAFE pathway as both $\text{TNF}\alpha^{-/-}$ and STAT-3 knockout mice failed to be protected with bradykinin and opioids. In contrast, adenosine could confer cardioprotection in $\text{TNF}\alpha^{-/-}$ but not in STAT-3^{-/-} mice, therefore suggesting a partial activation of the SAFE pathway with adenosine.

4.1. Adenosine as a postconditioning mimetic

Adenosine is one of many known autacoids that are released during the ischaemic postconditioning (Ovize et al., 2010). Interstitial levels of the nucleoside are increased during ischaemia and washed out during reperfusion (Schulz et al., 1995). Adenosine has multiple receptor subtypes (A_1 , A_{2A} , A_{2B} , and A_3) that are expressed in the myocardium and vasculature which have been exploited therapeutically with mixed results (Baxter et al., 2000, Burley and Baxter, 2009). Recent studies suggesting the endogenous activation of adenosine receptors by ischaemic postconditioning has renewed interest in the nucleoside as a cardioprotective agent. Hence, Kin et al. proved that postconditioning the isolated mouse heart delayed the intravascular adenosine washout and significantly reduced infarct size (Kin et al., 2005). They also employed an open-chest rat model where postconditioning produced an infarct limiting effect that was abolished in the presence of an A_2 , A_3 or non-selective AR antagonist but not with an A_1 AR antagonist. This study suggested that IPostC increases the endogenous adenosine levels that activate A_2 and A_3 AR subtypes to confer cardioprotection. Park et al also showed protection at reperfusion with an A_3 AR agonist (Park et al., 2006). The protective effects of A_{2A} and A_{2B} AR activation have specifically been demonstrated in mouse (A_{2A}) (Rork et al., 2008), rat (A_{2A} and A_{2B}) (Lasley et al., 2007), rabbit (A_{2B}) (Kuno et al., 2008), and dog (A_{2A}) (Glover et al., 2007). Previously, Yang et al. also provided evidence that the beneficial effect of postconditioning was dependant on adenosine receptors and used 8-(p-sulfophenyl)theophylline (SPT), a non-selective adenosine receptor

antagonist to block protection (Yang et al., 2005). Use of specific antagonists identified A_{2B} AR as a likely target for mediating protection.

Clinical trials with adenosine (Acute Myocardial Infarction Study of Adenosine – AMISTAD I and II) showed a significant reduction in infarct size but a weak clinical outcome in patients with a ST-segment elevation myocardial infarction undergoing reperfusion therapy (Ross et al., 2005, Mahaffey et al., 1999). At the end of AMISTAD II, insufficient data was obtained to substantiate adenosine as a viable treatment. However, fewer adverse effects were reported with the lower dosage of adenosine (70 µg/kg/min) and a larger scale study was proposed. Intravenous delivery of adenosine has been confounded by the side effects of hypotension and bradycardia. However, Takahama et al. have recently developed a novel technique whereby adenosine, encapsulated in polyethylene glycol-coated liposomes were infused at reperfusion and successfully reduced infarct size in ischaemic rats with no significant effects on the mean blood pressure or heart rate compared to free adenosine infusion (Takahama et al., 2009). The findings in our study support adenosine as a treatment option against reperfusion injury as a postconditioning mimetic. Although TNFα did not contribute to the cardioprotective effect of adenosine as a postconditioning mimetic, STAT-3 was critical to the postconditioning outcome. Interestingly, previous work in our laboratory showed that successful preconditioning with adenosine was also dependant on STAT-3 but not TNFα (Smith et al., 2002, Smith et al., 2004). Erk 1/2 or PKC activation have previously been linked to STAT-3 phosphorylation and may be involved (Frias et al., 2009, Xuan et al., 2001). It is possible that the signalling mechanism may involve adenosine, acting via its preferred adenosine receptor subtype to stimulate STAT-3 activation through Erk 1/2 or PKC. This may be followed by the subsequent translocation of STAT-3 from the cytosol to the mitochondria, as observed by the concomitant increase in STAT-3 phosphorylation levels. Another possibility would be that adenosine is part of the SAFE pathway and somehow, is an intermediate compound activated by TNFα but resides above STAT-3. Further experiments will be required to confirm this hypothesis.

4.2. Bradykinin and opioids activate the SAFE pathway in pharmacological postconditioning

Bradykinin and opioids are released from the myocardium after ischaemia. Preconditioning with these autacoids has shown to have a cardioprotective effect (Gross et al., 2004, Gross and Gross, 2006). Exogenous administration of bradykinin at reperfusion successfully limited reperfusion injury (Bell and Yellon, 2003). The B₂ receptor has been implicated as a likely candidate for triggering the cardioprotective event since B₂ receptor antagonists, icatibant and WIN64338 abrogated the postconditioning effect (Penna et al., 2007). This was confirmed in B₂ receptor knockout mice. An additive cardioprotective role for the B₁ receptor was suggested when ablation of the gene partly attenuated infarct size (Xi et al., 2008). The signalling mechanisms involved in the process are unclear but a role for the RISK pathway, eNOS and prostaglandin (PGI₂), has been suggested (Penna et al., 2008, Bell and Yellon, 2003).

With regards to opioids, Zatta et al. showed that IPostC preserved the myocardial opioid peptide concentrations during early reperfusion (Zatta et al., 2008). IPostC was abolished in the *in vivo* rat myocardium with the addition of non-selective agonist, naloxone or selective δ -opioid receptor antagonist, naltrindole (Jang et al., 2008). Interestingly, Chen et al. implicated the κ - but not δ -opioid receptor in morphine induced postconditioning of the isolated rat heart (Chen et al., 2008). There is a lack of knowledge of exogenous opioids given at reperfusion to limit reperfusion injury. Recently, morphine was given for 15 minutes at the onset of reperfusion after the ischaemic event and found to reduce infarct size comparable to ischaemic postconditioning (Huhn et al., 2010).

We applied a pharmacological postconditioning protocol on the isolated mouse heart whereby bradykinin or opioids were delivered via means of stuttered reperfusion. In contrast to the results obtained with adenosine, cardioprotection with these agents were dependant on both TNF α and STAT-3. Here, we provide evidence for the existence of prosurvival signalling pathways and implicate the SAFE pathway in postconditioning induced by bradykinin and DADLE.

4.3. STAT-3 activity in the mitochondria

The role for STAT-3 in ischaemic postconditioning has been demonstrated earlier in this thesis, whereby the genetic ablation of the gene completely abolished cardioprotection (section D). The recent discovery of STAT-3 in the mitochondria, phosphorylated at its serine residue suggested that the cardioprotective effect observed with STAT-3 in pre- and postconditioning may be related to its activation within the mitochondria rather than the nucleus (Wegrzyn et al., 2009). Hence, Wegrzyn et al. showed that STAT-3 was critical to the mitochondrial electron chain transport system and thus cellular homeostasis. Mice that do express STAT-3 in the heart exhibit defects in complexes I and II of the electron transport chain in the mitochondria which effect cellular homeostasis (Wegrzyn et al., 2009). The exact mechanism by which STAT-3 regulates complexes I and II remains to be determined. STAT-3 is known to regulate anti-apoptotic factor, Bcl-xL, located on the outer mitochondrial membrane (Lin et al., 2005, Zhang et al., 2007b). We observed an increase in the ratio of phosphorylated STAT-3 in the mitochondrial fraction, compared to the cytosolic fraction in hearts treated with bradykinin or DADLE. This may suggest a prosurvival signalling pathway involving STAT-3 translocation from the cytosol to the mitochondria. Interestingly, adenosine's cardioprotective effect was abolished in the absence of STAT-3. However, our western blot analysis did not reveal the expected increase in mitochondrial STAT-3 levels. It is possible that adenosine induced protection may require an additional mechanism of action for STAT-3 linked to its cytosolic activation or perhaps, translocation to the nucleus.

Our novel data show that pharmacological postconditioning with bradykinin and opioids activate STAT-3 via $\text{TNF}\alpha$, and thus the SAFE pathway, to confer protection against reperfusion injury (Figure 45). The low levels of STAT-3 detected in the mitochondria may suggest a "kinase-like" activity for the transcription factor in the signalling cascade. Further studies into the elucidation and dynamics of the multiple prosurvival pathways involved in ischaemic and pharmacological postconditioning is warranted as this may lead to potential clinical and novel therapeutic drug development. STAT-3 is involved in the regulation of genes in the nucleus which may encode proteins that are involved in the preservation of mitochondrial potential. However, its exact role in the mitochondria remains to be determined.

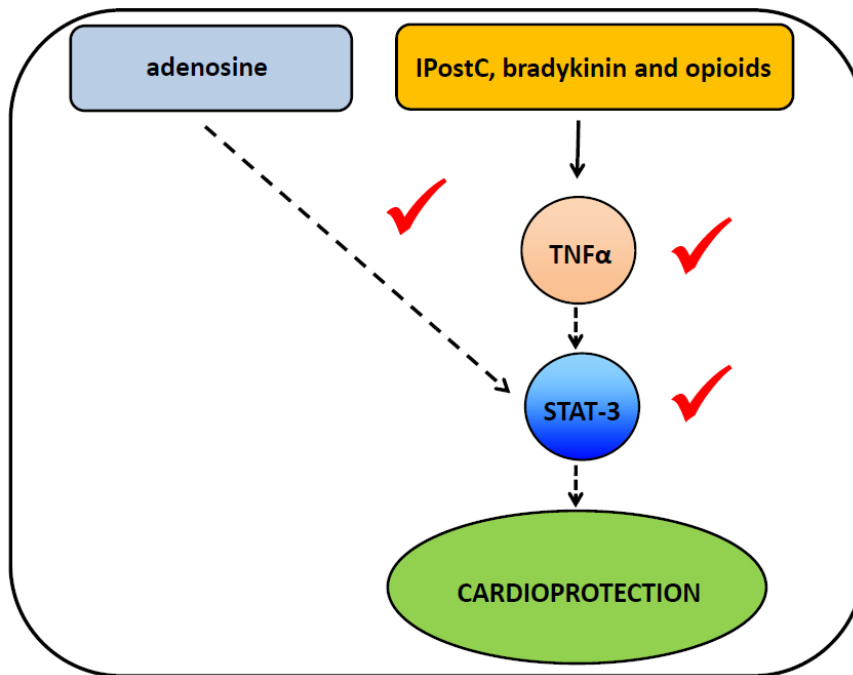


Fig 45. Summary of findings. Bradykinin and DADLE stimulated the activation of $\text{TNF}\alpha$ and subsequent phosphorylation of STAT-3 (SAFE pathway) leading to cardioprotection. Pharmacological postconditioning with adenosine required STAT-3 but not $\text{TNF}\alpha$.

F. ROLE OF STAT-3 IN S1P/ HDL INDUCED CARDIOPROTECTION

Part of this work was done in collaboration with Dr Miguel Frias and Prof Richard James of University of Geneva Hospital, Switzerland

1.0. Introduction

Lipoproteins are macromolecular structures that are composed of free cholesterol, a core of cholesteryl esters, triglycerides and surrounded by an envelope of phospholipids (Figure 46) (Libby, 2008). There are five major classes of lipoproteins, chylomicrons, Very Low Density Lipoprotein (VLDL), Intermediate Density Lipoprotein (IDL), Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL). HDL is the smallest of the lipoproteins but are also the most dense as they are enriched with apolipoproteins, Apo A-I and Apo A-II. These apolipoproteins assist HDL in the removal of excess LDL-cholesterol via the reverse cholesterol transport. HDL is considered as the 'good cholesterol' and elevating its levels with statins, fibrates and other lipid-lowering drugs are used to treat patients with coronary artery disease (Brown et al., 2006, Otvos et al., 2006, 1984). HDL also possesses many other cardiovascular benefits due to its anti-inflammatory, anti-oxidant, anti-apoptotic and vasodilatory properties (Barter et al., 2004, Mineo et al., 2006, Argraves and Argraves, 2007).

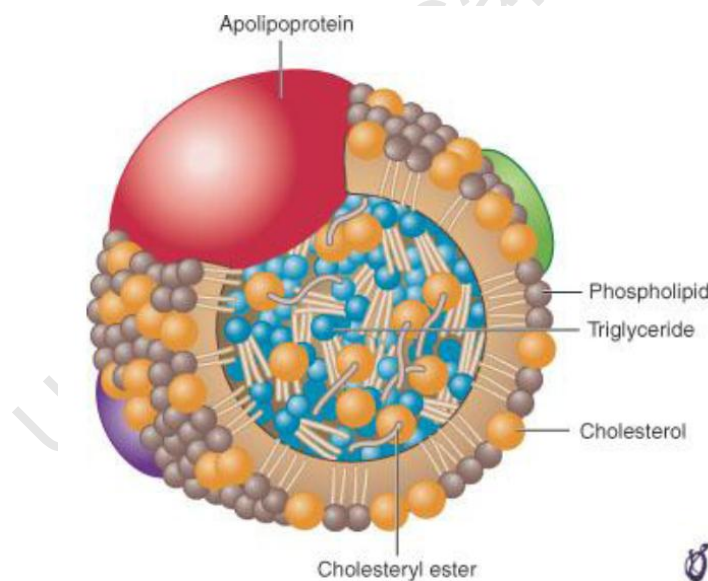


Fig 46. Structure of lipoproteins. Phospholipids form the envelope of the structure where the polar groups are directed towards the aqueous environment. Free cholesterol is located within the phospholipid layer. Cholesteryl esters and triglycerides make up the core of the lipoprotein. Apolipoproteins are involved in the secretion of the lipoprotein, afford structural integrity, and function as cofactors for enzymes or as ligands for different receptors. (From Braunwald's Heart Disease: A textbook of cardiovascular medicine. Image copyright 2008 by Saunders, an imprint of Elsevier (Libby, 2008)).

In a setting of ischaemia/ reperfusion injury, HDL reduced harmful TNF α levels when administered either prior or after the ischaemic insult (Calabresi et al., 2003). Recently, S1P has been identified as a constituent of HDL and has been suggested to be responsible for mediating some of its protective effects (Theilmeier et al., 2006, Kimura et al., 2001, Okajima, 2002, Frias et al., 2009, Frias et al., 2010). Levkau and colleagues demonstrated that HDL and S1P protected the *in vivo* mouse myocardium from ischaemia/ reperfusion injury via nitric oxide and S1PR₃ (Theilmeier et al., 2006). Frias et al. showed native and reconstituted HDL with a varied composition of S1P protected cardiomyocytes against doxorubicin induced apoptosis via S1PR₂ but not S1PR₁ and S1PR₃ (Frias et al., 2010). In a separate study, this group also implicated a role for prosurvival factors STAT-3 and ERK1/2 in HDL induced cardioprotection (Frias et al., 2009).

The addition of S1P or sphingosine after forty minutes of ischaemia significantly improved left ventricular developed pressure and elevated depleted levels of Akt, a prosurvival kinase of the well established Reperfusion Injury Salvage Kinase (RISK) pathway (Vessey et al., 2008a, Tsang et al., 2004). The combined addition of the sphingolipids extended the protection after more prolonged periods of ischaemia. This additive effect is explained by the different mechanisms of each agent. S1P functions through a G-coupled protein receptor pathway and sphingosine through a cyclic nucleotide pathway (Vessey et al., 2008b). Similar to preconditioning, IPostC was abolished in SPK1^{-/-} mice (Jin et al., 2008). These studies suggest that the signalling mechanisms involved in pharmacologically induced pre- and postconditioning with S1P may be closely related. Recently, a novel cardiac prosurvival path named the Survivor Activating Factor Enhancement (SAFE) pathway and involving the activation of tumor necrosis factor alpha (TNF α) and the transcription factor signal transducer and activator of transcription-3 (STAT-3) has been delineated (see review (Lecour, 2009)). In this thesis, we have demonstrated the role of the SAFE pathway in both ischaemic and pharmacological postconditioning such as bradykinin and opioids. Although S1P is capable of activating STAT3 in cardiomyocytes (Frias et al., 2009), the necessity for S1P to activate this path in order to confer protection against ischaemia/ reperfusion injury is unknown.

We thus hypothesized that HDL/ S1P protects the heart against reperfusion injury via the activation of the SAFE pathway and we propose to investigate a possible interplay between the two powerful cell survival pathways, namely the SAFE and the RISK path (Figure 47.).

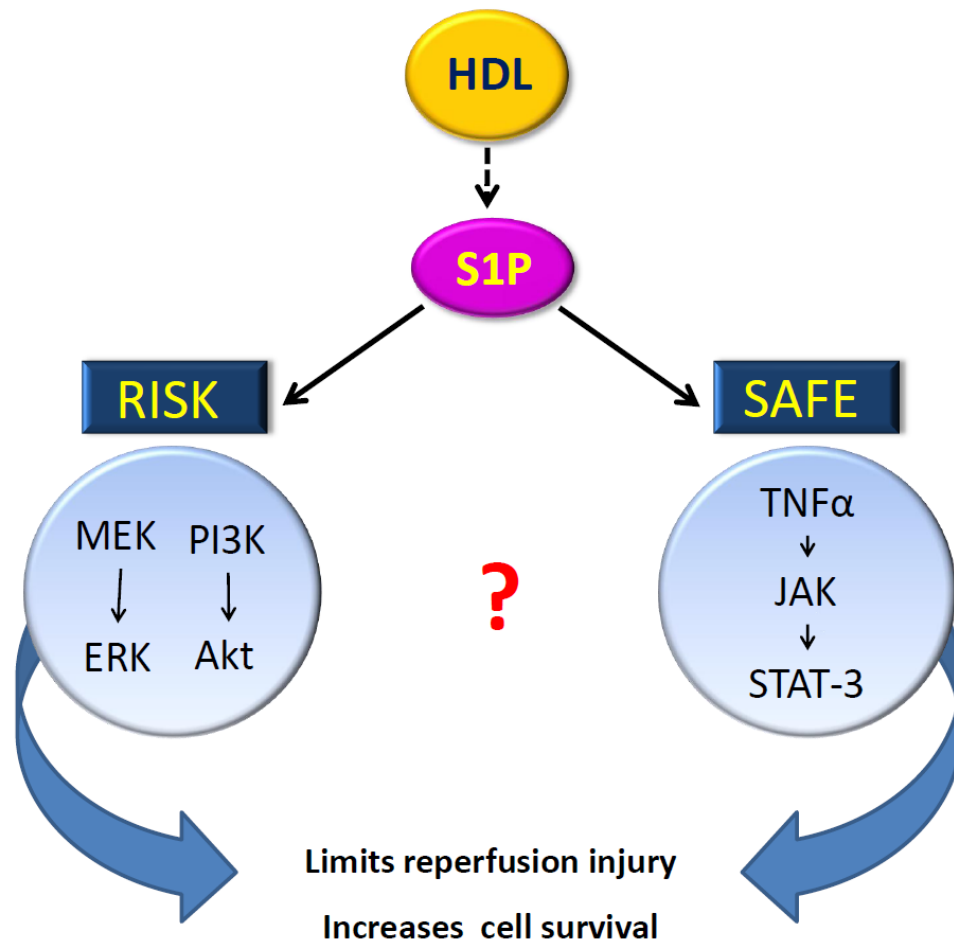


Fig 47. Schematic of proposed hypothesis. The cardioprotective properties of HDL may be attributed to S1P and activation of the SAFE and RISK pathways. *HDL*, High Density Lipoprotein; *S1P*, Sphingosine 1-Phosphate; *RISK*, Reperfusion Injury Salvage Kinase; *MEK*, Mitogen activated protein kinase Extracellular regulated Kinase; *ERK*, Extracellular Regulated Kinase; *PI3K*, Phosphoinositol 3-Kinase; *SAFE*, Survivor Activation Factor Enhancement; *TNFα*, Tumour Necrosis Factor alpha; *JAK*, Janus Kinase; *STAT-3*, Signal Transducer and Activator of Transcription-3.

2.0. Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85 (23), revised 1996). All procedures were approved by the Faculty of Health Sciences

Animal Ethics Committee, University of Cape Town under AEC reference number 009/052. Cardiomyocyte-specific STAT-3-deficient mice (STAT-3^{-/-}) from C57Black6 background were created in our facilities by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre recombinase mice, as previously described (Smith et al., 2004). Homozygous TNF α -deficient mice were also used. All mice were 14–16 weeks of age.

2.1.1. Perfusion of mouse hearts

The Langendorff perfusion model was discussed earlier in Section C. All hearts were subjected to 20 min of stabilization, 35 min global ischaemia and 45 min reperfusion (Figure 48). Hearts were treated with HDL (200 μ g/ml) or S1P (10 nM) for the first 7 minutes of reperfusion. To determine the involvement of the SAFE and RISK pathways, AG490 (AG), an inhibitor of the JAK/STAT pathway (100 nM), Wortmannin (W), a Phosphatidylinositol-3 Kinase (PI-3K)/Akt (100 nM) inhibitor, or PD98059 (PD), an inhibitor of the MEK/ERK pathway (100 nM) was perfused together with S1P or alone for 15 min after ischaemia.

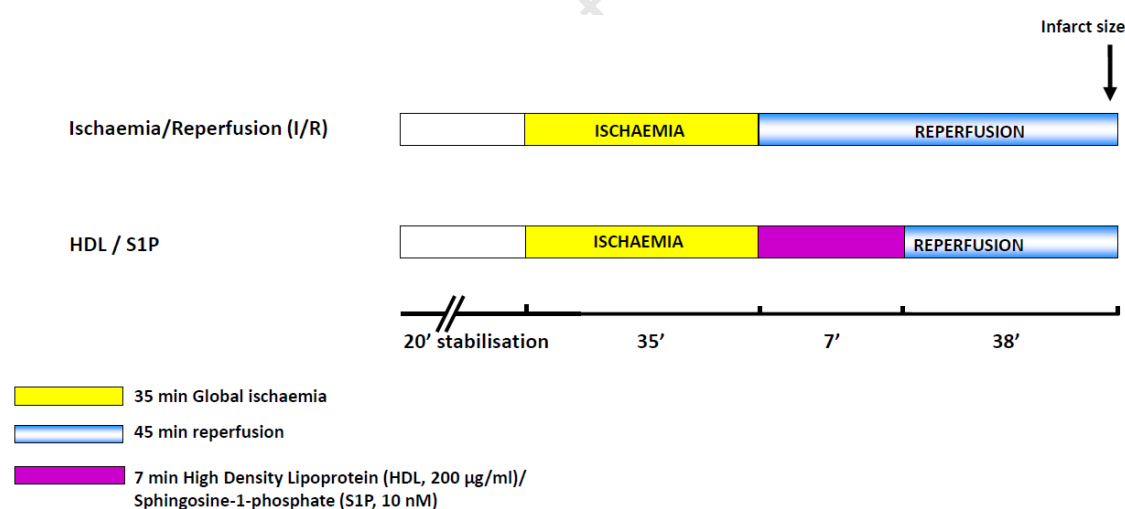


Fig 48. Schematic representation of the experimental protocol. Isolated hearts were subjected to 35 min ischaemia followed by 45 min reperfusion. At the onset of reperfusion, HDL (200 μ g/ml) or S1P (10 nM) was given for 7 min. *HDL*, high density lipoprotein; *S1P*, sphingosine-1-phosphate

2.1.2. Infarct size assessment

At the end of the experiment, hearts were stained with triphenyltetrazolium chloride to measure infarct size, as described previously (Smith et al., 2002). Hearts were then frozen overnight and sectioned into 1.5 mm slices. The slices were tightly placed between two glass plates at a distance of 0.5 mm apart and digitally scanned. A minimum of 6 hearts per group was used in this study. Infarct size was calculated using computerized planimetry (Planimetry+, Boreal Software, Norway).

2.2. Western Blot Analysis

Control or treated hearts were subjected to 20 min stabilization, 35 min global ischaemia followed by 15 min reperfusion. At this point, hearts were snap frozen in liquid nitrogen and stored at -80°C. Cytosolic and nuclear or mitochondrial extractions were performed as previously described (Lecour et al., 2005b). Phosphorylated and total levels of STAT-3, Akt, ERK, and FOXO-1, were analysed by sodium dodecyl sulphate polyacrylamide gel immunoelectrophoresis (SDS-PAGE). Equal loading was verified by measuring levels of β -actin (cytosol/nucleus) or VDAC (mitochondria). Densitometry analysis was carried out using Image J software. A minimum of four hearts per group were used.

2.3. Chemicals and pharmacological agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich chemicals, Germany. HDL was extracted from the blood of healthy patients, as described earlier in section C. Western blot antibodies were obtained from Cell Signaling Technologies, USA.

2.4. Statistical analysis

Data are expressed as mean values \pm S.E.M and were analysed by performing multiple group comparisons using one way ANOVA and followed by Student-Newman-Keuls post hoc test or T-test (Graph Pad Instat). A value of $P < 0.05$ was considered to be statistically significant.

3.0. Results

3.1. TNF α and STAT-3 are essential for HDL induced protection

HDL was given following global ischaemia for 7 min of reperfusion in both TNF α ^{-/-} and STAT-3^{-/-} mouse hearts. In the TNF-WT, HDL significantly reduced infarct size ($25.8 \pm 3.4\%$) compared to the ischaemic control (IC) (Figure 49, $40.0 \pm 2.5\%$ for IC, $P < 0.05$). This beneficial effect was not observed in the TNF α ^{-/-} hearts ($41.6 \pm 2.2\%$ for HDL vs $38.3 \pm 1.5\%$ for IC, $P = \text{ns}$). Similarly, HDL protected the STAT-3-WT hearts ($23.3 \pm 2.0\%$ for HDL vs $39.8 \pm 2.0\%$ for IC, $P < 0.05$) but not the STAT-3 knockout hearts ($37.1 \pm 2.2\%$ for HDL vs $40.0 \pm 2.1\%$ for IC, $P = \text{ns}$).

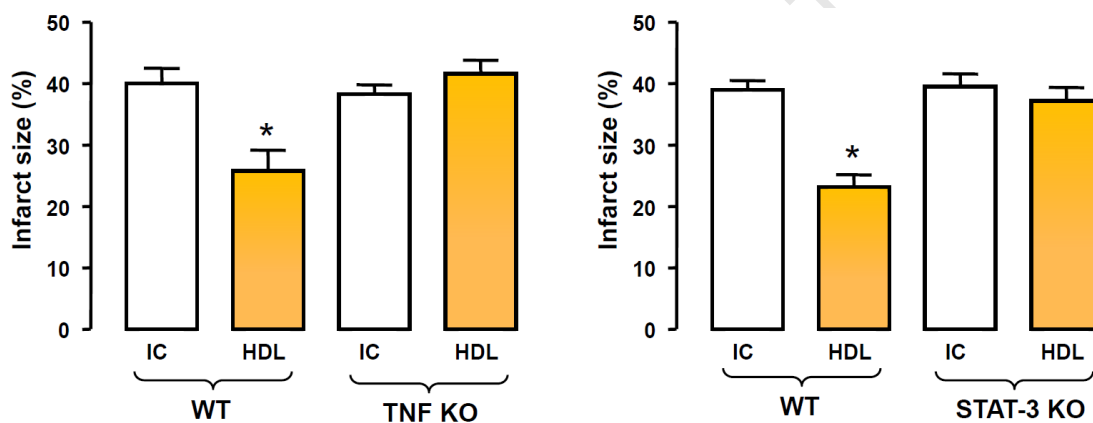


Fig 49. HDL induced cardioprotection is dependent on the TNF α and STAT-3. The cardioprotective effect of HDL is abolished in TNF α deficient and cardiomyocyte-specific STAT-3 knockout mice subjected to ischaemia/reperfusion in the isolated mouse heart model. * $P < 0.05$ vs control ($n = 6$ per group). *WT*, wildtype; *KO*, knockout; *TNF*, Tumour Necrosis Factor α ; *STAT-3*, Signal transducer and activator of transcription-3; *HDL*, High Density Lipoprotein

3.2. HDL activated mitochondrial STAT-3

Phosphorylated levels of STAT-3 were measured following 7 min of HDL treatment during reperfusion in STAT-3 WT hearts (Figure 50). HDL decreased STAT-3 phosphorylation in the cytosol (In arbitrary units (AU), 3.4 ± 0.3 AU for IC and 2.0 ± 0.4 AU for HDL, $p < 0.05$) and this effect was associated with an increase of phosphorylated STAT-3 in the nucleus (1.6 ± 0.0 AU for IC and 2.4 ± 0.0 AU for AG, $p < 0.05$).

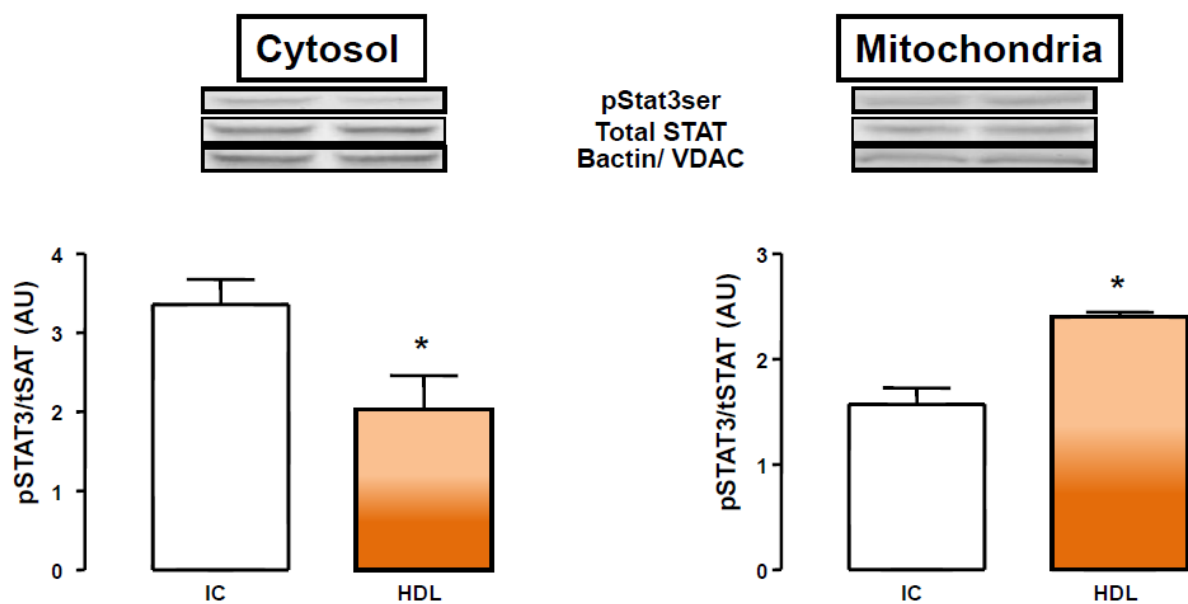


Fig 50. Phosphorylated STAT-3 translocated to the mitochondria following HDL treatment. Phosphorylated STAT-3 levels are decreased in the cytosol and increased in the mitochondrial fraction in the presence of HDL. * $P < 0.05$ vs control ($n = 4$). IC, ischaemic control; STAT-3, Signal transducer and activator of transcription-3; HDL, High Density Lipoprotein.

3.3. TNF α and STAT-3 are essential for S1P induced cardioprotection

To investigate the role of the SAFE pathway in S1P induced cardioprotection, the infarct size was measured in the isolated perfused hearts of TNF α and STAT-3 knockout mice and their respective littermate controls following S1P administration and subsequent reperfusion (Figure 51). S1P reduced the infarct size in the TNF-WT from $32 \pm 2.1\%$ in the ischaemic control (IC) group to $12.1 \pm 2.5\%$ for S1P ($P < 0.05$, Fig. 1). In contrast, S1P failed to reduce the infarct size in TNF $\alpha^{-/-}$ ($34.1 \pm 2.0\%$ for S1P vs $31.1 \pm 1.8\%$ for IC, $P = \text{ns}$). Similarly, S1P significantly reduced infarct size in STAT-3-WT hearts ($12.1 \pm 2.5\%$ for S1P vs $32.2 \pm 2.1\%$ for IC, $P < 0.05$) but not in the STAT3 $^{-/-}$ ($34.2 \pm 4.3\%$ for S1P vs $30.2 \pm 2.7\%$ for IC, $P = \text{ns}$).

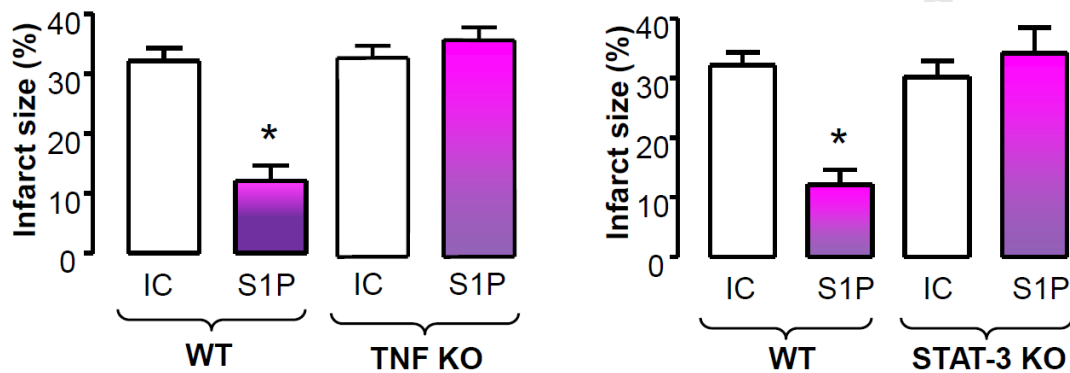


Fig 51. S1P requires TNF α and STAT-3 to confer cardioprotection. The cardioprotective effect of S1P is abolished in TNF α deficient and cardiomyocyte-specific STAT-3 knockout mice subjected to ischaemia/reperfusion in the isolated mouse heart model. * $P < 0.05$ vs control ($n = 6$ per group). *WT* wildtype, *KO* knockout, *TNF* Tumour Necrosis Factor α , *STAT-3* Signal transducer and activator of transcription-3, *S1P* sphingosine 1-phosphate

3.4. Pharmacological inhibition of STAT-3 abrogates the protective effect of S1P

To further confirm the involvement of STAT-3 in S1P induced protection against reperfusion injury, we measured the infarct size in STAT-3-WT hearts perfused with an inhibitor for the JAK/STAT-3 pathway (AG490) during the first 15 min of reperfusion (Figure 52). Addition of AG490 (AG) abolished the infarct-sparing effect of S1P ($34.1 \pm 3.3\%$ for S1P+AG vs $17.5 \pm 2.2\%$ for S1P, $P < 0.05$). There was no significant difference in infarct size between the IC and AG groups ($41.8 \pm 2.7\%$ for IC and $31.1 \pm 5.1\%$ for AG).

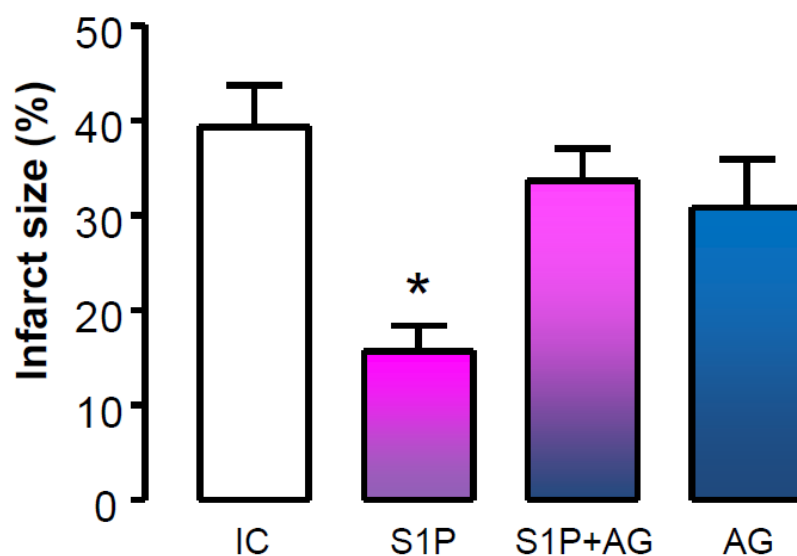


Fig 52. JAK/STAT-3 inhibitor, AG490 blocks S1P induced protection. Addition of the STAT-3 inhibitor AG490 (100 nmol/L) with S1P abolished the infarct sparing effect of S1P. * $P < 0.05$ vs control ($n = 6$). IC, ischaemic control; STAT-3, Signal transducer and activator of transcription-3; S1P, sphingosine 1-phosphate; AG, AG490

3.5. S1P increases nuclear phosphorylated STAT-3

Furthermore, the levels of STAT-3 were measured after 15 min of reperfusion in both cytosolic and nuclear fractions. S1P treatment was associated with a decrease of cytosolic phosphorylated STAT-3 (from 1.2 ± 0.1 AU for IC to 0.8 ± 0.1 AU for S1P, $P < 0.05$, Figure 53). Similarly, addition of AG490 reduced STAT-3 levels alone, and in tandem with S1P (0.8 ± 0.1 AU for S1P+AG and 0.6 ± 0.2 AU for AG). In contrast, S1P treatment was associated with an increase of phosphorylated STAT-3 in the nucleus (0.5 ± 0.1 AU for IC to 1.0 ± 0.2 AU for S1P, $P < 0.05$). This effect was abolished with addition of AG490 (0.4 ± 0.1 AU for S1P+AG) and similarly, AG alone exhibited only basal levels of STAT-3 comparable to the IC group (0.5 ± 0.1 AU).

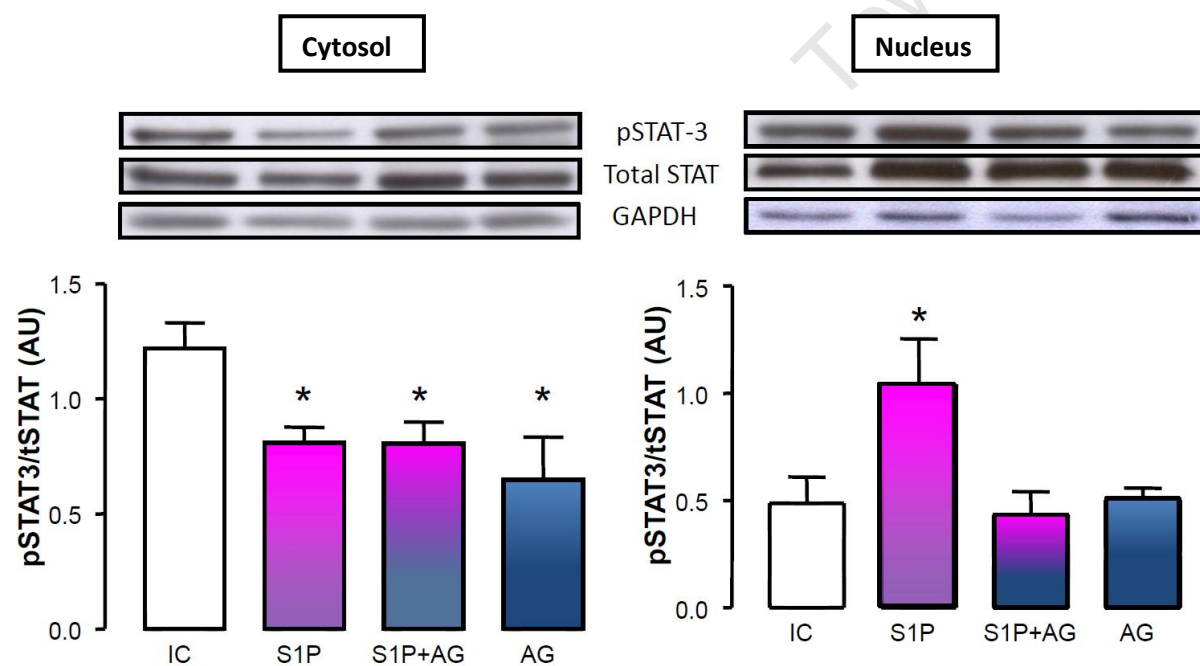
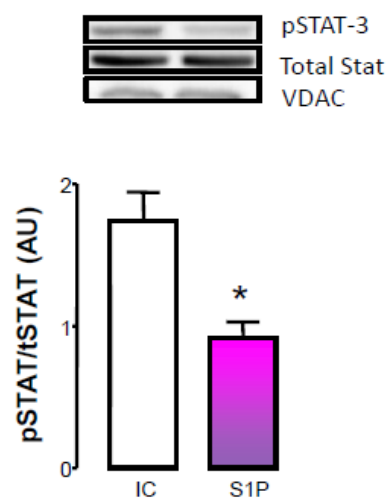
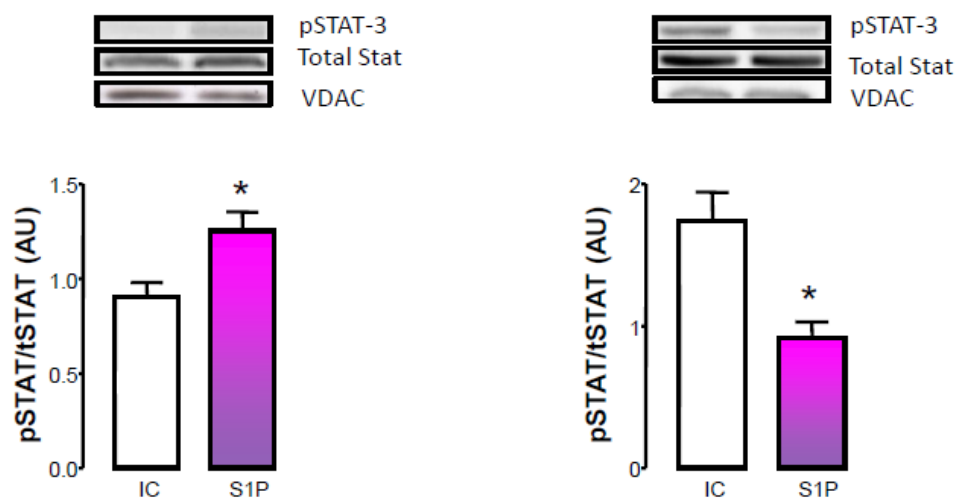


Fig 53. S1P induces nuclear translocation of phosphorylated STAT-3. S1P decreased phosphorylation of cytosolic STAT-3 and increased levels of phosphorylated STAT-3 in the nucleus. * $P < 0.05$ vs control ($n = 4$). IC, ischaemic control; STAT-3, Signal transducer and activator of transcription-3; S1P, sphingosine 1-phosphate, AG, AG490.

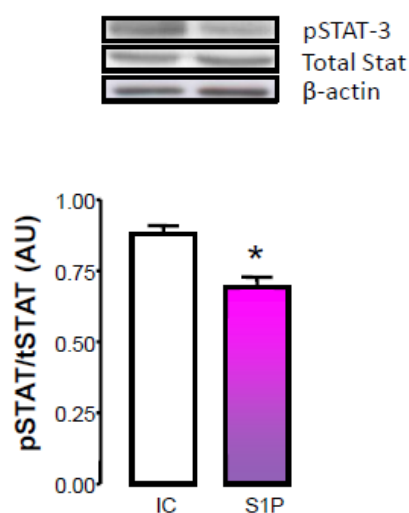
3.6. S1P activates mitochondrial STAT-3

We compared the phosphorylated levels of STAT-3 in cytosolic and mitochondrial fractions in S1P treated hearts after 7 min or 15 min of reperfusion. Phosphorylated STAT-3 levels were significantly increased with S1P in the mitochondria after 7 min of reperfusion (0.9 ± 0.1 AU for IC to 1.3 ± 0.1 AU for S1P, $P < 0.05$, Figure 54a). In contrast, there was a marked reduction in phosphorylated STAT-3 levels observed with S1P in the mitochondria after 15 min of reperfusion (1.7 ± 0.2 AU for IC to 0.9 ± 0.1 AU for S1P, $P < 0.05$, Figure 54b). In the cytosolic fraction, pretreatment with S1P was associated with a decrease in STAT-3 phosphorylation at both 7 min and 15 min of reperfusion (0.9 ± 0.0 AU for IC to 0.7 ± 0.0 AU for S1P, $P < 0.05$, Figure 54c; 1.5 ± 0.2 AU for IC to 0.7 ± 0.1 AU for S1P $P < 0.05$, Figure 54d), respectively.

a) 7 min mitochondrial fraction b) 15 min mitochondrial fraction



c) 7 min cytosol fraction



d) 15 min cytosol fraction

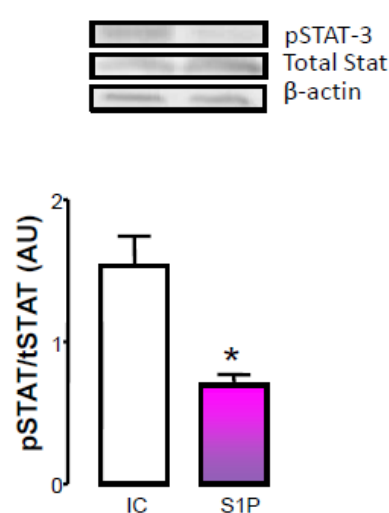


Fig 54. Mitochondrial translocation of STAT-3 is induced by S1P. **a** Increased phosphorylation of STAT-3 in the mitochondria at 7 min reperfusion *P < 0.05 vs control (n = 4). **b** Representative western blot demonstrating decreased levels of phosphorylated STAT-3 in the mitochondria at 15 min reperfusion *P < 0.05 vs control (n = 4). **c** Phosphorylated STAT-3 are decreased in the cytosol at 7 min reperfusion *P < 0.05 vs control (n = 4). **d** Western blot analysis revealed a decreased levels of phosphorylated STAT-3 in the cytosol at 15 min reperfusion *P < 0.05 vs control (n = 4). IC ischaemic control, S1P sphingosine 1-phosphate

3.7. Inhibition of Akt but not ERK abrogates the protective effect of Sphingosine-1-Phosphate

To investigate the involvement of the RISK pathway in S1P induced protection against reperfusion injury, infarct size was measured in STAT-3-WT hearts perfused with either wortmannin, an inhibitor of the PI-3 kinase/Akt pathway (W), or PD98059, an inhibitor of Mek/Erk1/2 (PD). Addition of wortmannin abolished the infarct-sparing effect of S1P ($37.1 \pm 4.8\%$ for S1P+W vs $17.5 \pm 2.2\%$ for S1P, $P < 0.05$, Figure 55). There was no significant difference in infarct size between the IC and W groups ($41.8 \pm 2.7\%$ for IC and $36.1 \pm 5.0\%$ for W, $P = \text{ns}$). Interestingly, addition of PD did not significantly abolish the protective effect of S1P ($22.3 \pm 6.1\%$ for S1P+PD vs IC, $P < 0.05$). PD alone, had no effect on infarct size ($30.3 \pm 10.7\%$ vs IC, $P = \text{ns}$).

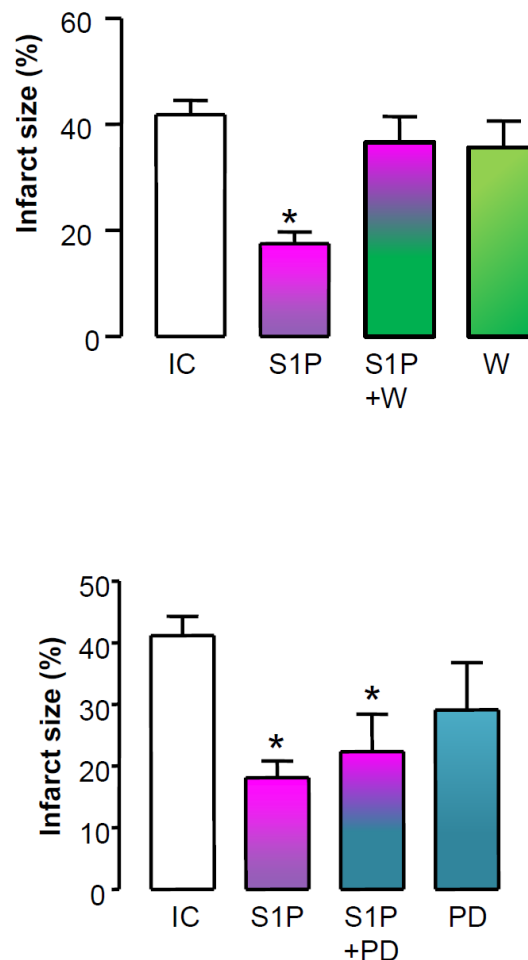


Fig 55. Akt but not, Erk inhibitors abrogate the protective effect of S1P in WT mice. Inhibiting the activation of Akt (by wortmannin) and Erk (by PD98059) during early reperfusion abrogates the protective effect of S1P in wildtype mice. * $P < 0.05$ vs control ($n = 6$ per group). IC ischaemic control, S1P sphingosine 1-phosphate, W wortmannin, PD PD98059

3.8. Levels of Akt, Erk and STAT-3 in S1P treated hearts

We examined the cytosolic and nuclear levels of Akt, Erk and STAT-3 during the first 15 min of reperfusion after S1P administration. Phosphorylated Akt levels were significantly decreased in the cytosolic S1P group compared to the IC group (2.8 ± 0.5 AU for IC vs 1.4 ± 0.3 AU for S1P, $P < 0.05$, Figure 56a). In contrast, nuclear levels of phosphorylated Akt were increased (1.1 ± 0.1 AU for IC vs 1.9 ± 0.4 AU for S1P, $P < 0.05$). Wortmannin and PD98059, alone showed reduced levels of phosphorylated Akt in both cytosolic (1.2 ± 0.2 AU for W, and 1.0 ± 0.2 AU for PD vs IC, $P < 0.05$) and nuclear fractions (1.4 ± 0.3 AU for W and 1.0 ± 0.2 AU for PD vs IC, $P = \text{ns}$). Similarly, co-treatment of S1P with Akt or Erk inhibitors exhibited lower levels of Akt in both the cytosolic (1.5 ± 0.6 AU for S1P+W; 1.2 ± 0.4 AU for S1P+PD vs IC, $P < 0.05$) and nuclear fractions (1.2 ± 0.3 AU for S1P+W and 1.1 ± 0.2 AU for S1P+PD vs IC, $P = \text{ns}$).

Cytosolic levels of phosphorylated Erk were slightly reduced with S1P treatment (1.7 ± 0.1 AU for IC vs 1.4 ± 0.0 AU for S1P, $P = \text{ns}$, Figure 56b) but increased in the nuclear fraction, compared to their respective control (1.1 ± 0.2 AU for IC vs 1.8 ± 0.4 AU for S1P, $P < 0.05$). There was no significant change in phosphorylated Erk levels observed in the wortmannin (alone) treated groups from either cytosolic (1.3 ± 0.2 AU for W vs IC, $P = \text{ns}$) or nuclear fraction (1.5 ± 0.2 AU for W vs IC, $P = \text{ns}$). However, PD98059 control group was significantly decreased in the cytosol compared to the ischaemic control (1.0 ± 0.1 AU for PD vs IC, $P < 0.05$). This effect was not observed in the nuclear fraction (1.0 ± 0.2 AU for PD vs IC, $P = \text{ns}$). Co-treatment of S1P and W did not significantly alter Akt levels in either cytosolic (1.4 ± 0.2 AU for S1P+W vs IC, $P = \text{ns}$) or nuclear fractions (1.5 ± 0.1 AU for S1P+W, $P = \text{ns}$). S1P, together with PD, exhibited significantly lower levels of cytosolic phosphorylated Erk levels compared with the IC group (1.1 ± 0.1 AU for S1P+PD vs IC, $P < 0.05$). In contrast, phosphorylated levels of Akt obtained from the S1P and PD treated group were at basal levels in the nuclear fraction (1.1 ± 0.1 AU for S1P+PD vs IC, $P = \text{ns}$).

Cytosolic STAT-3 levels were decreased in the S1P group compared to the IC group, as previously reported in section 3.5 (2.7 ± 0.3 AU for IC to 1.1 ± 0.1 AU for S1P, $P < 0.05$, Figure 56c). In contrast, phosphorylated levels of nuclear STAT-3 were increased for the S1P group compared to the IC group from 1.1 ± 0.2 AU for IC

to 2.1 ± 0.4 AU for S1P, $P < 0.05$). Both W and PD inhibitors significantly decreased levels of phosphorylated STAT-3 in the cytosolic fraction (1.4 ± 0.3 AU for W and 1.1 ± 0.2 AU for PD vs IC, $P < 0.05$). There was no change in nuclear levels of phosphorylated STAT-3 in either W or PD group (1.5 ± 0.2 AU for W and 1.0 ± 0.2 AU for PD vs IC, $P = \text{ns}$). The S1P+W and S1P+PD cytosolic groups showed significantly decreased phosphorylated STAT-3 levels (1.0 ± 0.2 AU for S1P+W and 1.4 ± 0.3 AU for S1P+PD vs IC, $P < 0.05$) but there was not change observed in the nuclear groups compared with their respective IC groups (1.5 ± 0.1 AU for S1P+W and 1.1 ± 0.1 AU for S1P+PD vs IC, $P = \text{ns}$).

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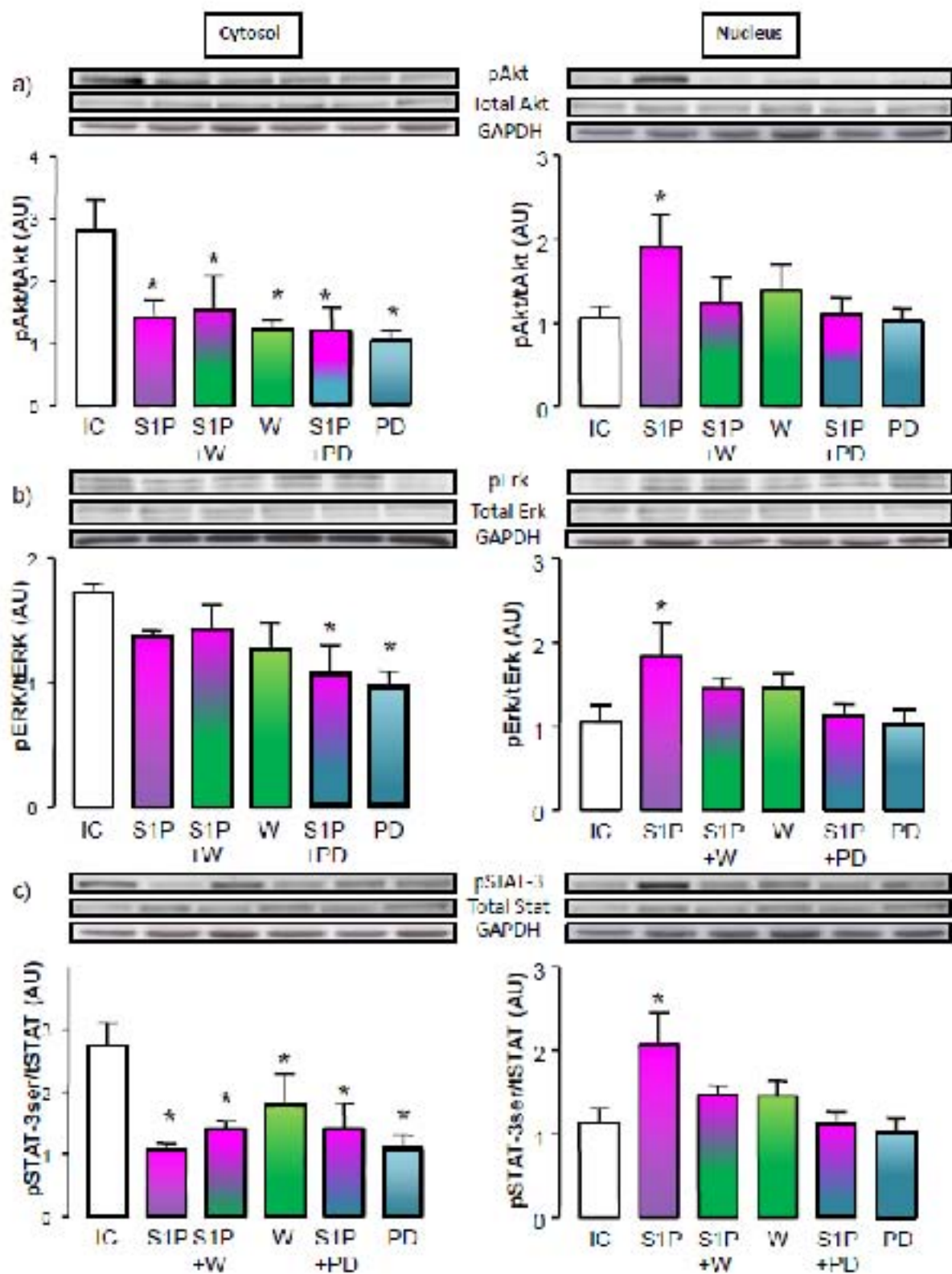


Fig 56. Phosphorylation of Akt, Erk and STAT-3 in WT mice. Bar graphs derived from western blots of cytosolic (left) and nuclear (right) fractions demonstrating phosphorylation of Akt, Erk and STAT-3 after 15 min reperfusion in S1P treated hearts. * $P < 0.05$ vs control ($n = 4$). IC, ischaemic control; S1P, sphingosine 1-phosphate; W, wortmannin; PD, PD98059; STAT-3, Signal transducer and activator of transcription-3

3.9. Levels of Akt and Erk in STAT-3^{-/-}

Cytosolic and nuclear levels of phosphorylated Akt and Erk were measured in cardiac deficient STAT-3 mice hearts treated with S1P (Figure 57). Phosphorylated Akt levels were not significantly altered between the IC and S1P treated groups in both the cytosolic (1.3 ± 0.1 AU for IC and 1.3 ± 0.1 AU for S1P, $P = \text{ns}$) and nuclear fractions (1.1 ± 0.3 AU for IC and 1.0 ± 0.1 AU for S1P, $P = \text{ns}$). Similarly, there was no increase in both cytosolic and nuclear phosphorylated Erk levels in the S1P group compared to the IC group (2.2 ± 0.5 AU for cytosolic IC and 2.0 ± 0.4 AU for cytosolic S1P; 1.6 ± 0.2 AU for nuclear IC and 1.6 ± 0.2 AU for nuclear S1P, $P = \text{ns}$).

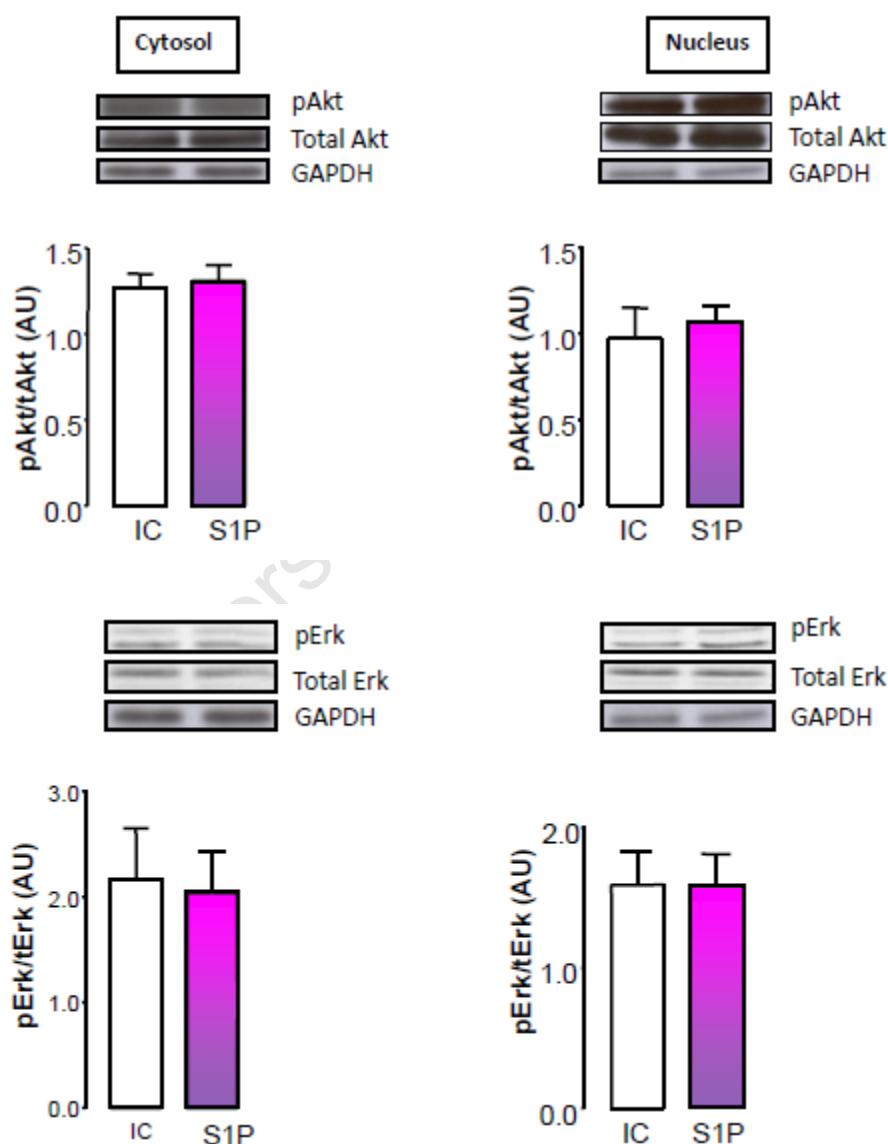


Fig 57. Phosphorylation of Akt, Erk in cardiomyocyte specific STAT-3 knockout mice. The absence of STAT-3 does not alter phosphorylated Akt and Erk levels in cytosolic and nuclear fractions. $P = \text{ns}$ ($n = 4$). *IC*, ischaemic control; *S1P*, sphingosine 1-phosphate

3.10. Levels of FOXO-1 in WT and STAT-3^{-/-}

FOXO-1 is a known downstream target of Akt (Matsuzaki et al., 2003, Ni et al., 2007) and its role was examined in S1P induced cardioprotection. In WT mice, treatment with S1P was associated with a decrease in cytosolic phosphorylated FOXO-1 levels compared to the IC group (1.8 ± 0.1 AU for IC to 1.2 ± 0.1 AU for S1P, $P < 0.05$, Figure 58a). When Akt and Erk were inhibited with W or PD, S1P also decreased phosphorylated FOXO-1 in the cytosol (1.4 ± 0.1 AU for S1P+W, 0.9 ± 0.1 AU for W, 0.8 ± 0.1 AU for S1P+PD and 0.9 ± 0.2 AU for PD vs IC, $P < 0.05$). In contrast, S1P treatment increased phosphorylated FOXO-1 levels in the nuclear fraction (0.8 ± 0.1 AU for IC to 1.1 ± 0.1 AU for S1P, $P < 0.05$). Wortmannin and PD98059 inhibited this effect (0.8 ± 0.1 AU for S1P+W and 0.7 ± 0.1 AU for S1P+PD vs IC, $P = \text{ns}$). Neither W nor PD levels, alone affected FOXO-1 levels compared to the control (0.8 ± 0.1 AU for W and 0.6 ± 0.1 AU for PD vs IC, $P = \text{ns}$). In STAT-3^{-/-} mice treated with S1P, there was no significant change in phosphorylated FOXO-1 levels for both cytosolic (1.1 ± 0.1 AU for IC to 0.9 ± 0.1 AU for S1P, $P = \text{ns}$, Figure 58b) and nuclear (1.0 ± 0.3 AU for IC to 1.2 ± 0.2 AU for S1P, $P = \text{ns}$) between the S1P treated group and IC group.

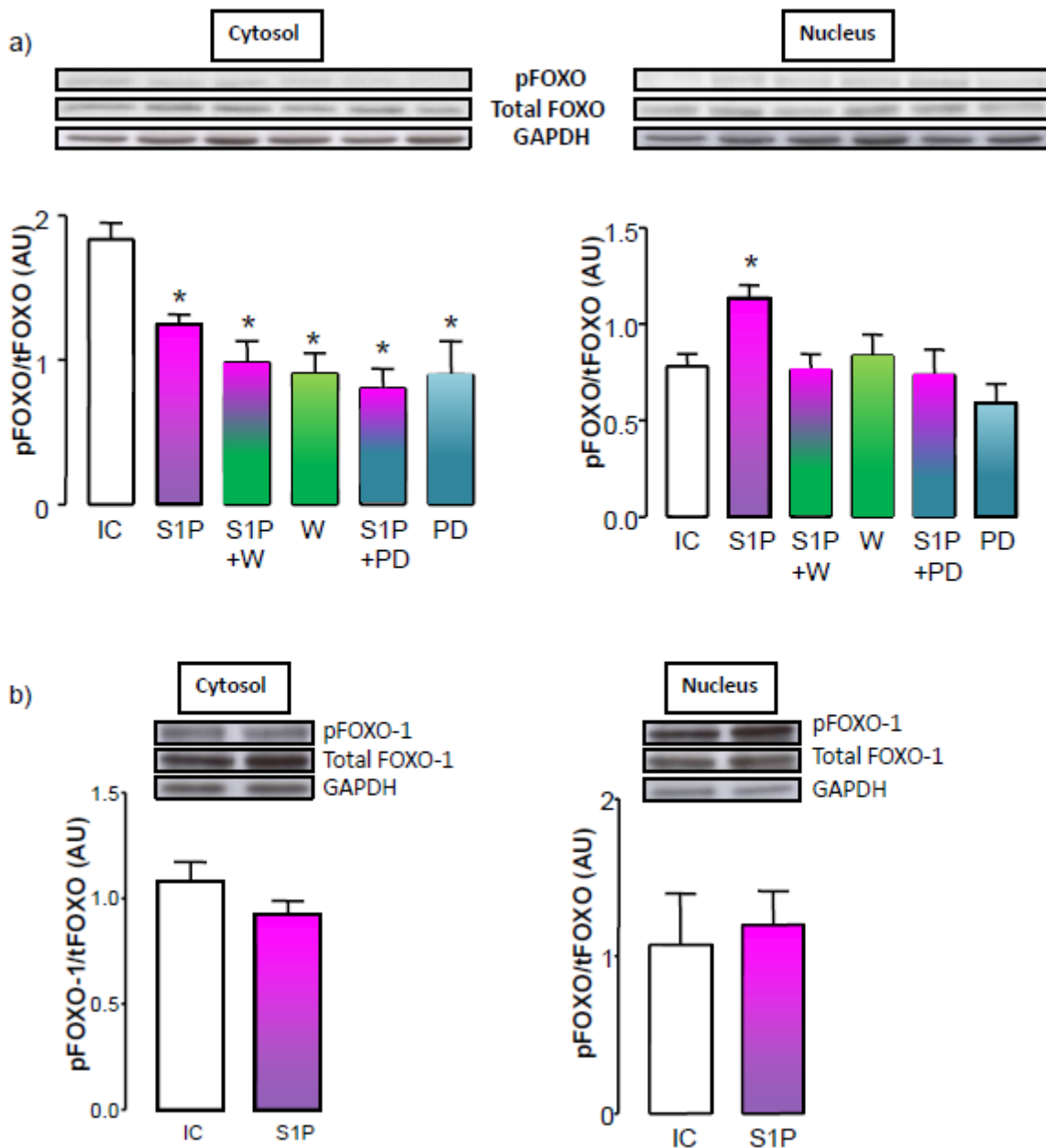


Fig 58. Phosphorylation of FOXO-1 in WT and STAT-3^{-/-} mice. **a** Bar graphs derived from western blot of cytosolic and nuclear fraction demonstrating phosphorylation of FOXO-1 after 15 min reperfusion in S1P treated hearts from WT mice. *P < 0.05 vs control (n = 4). **b** Phosphorylated FOXO-1 levels did not differ in the cytosolic and nuclear fraction in S1P treated hearts from STAT-3^{-/-} mice. P=ns. IC, ischaemic control; S1P, sphingosine 1-phosphate; W, wortmannin; PD, PD98059; STAT-3, Signal transducer and activator of transcription-3

4.0. Discussion

Our data show that exogenous HDL or its cardioprotective component, sphingosine-1-phosphate given at the onset of reperfusion confer cardioprotective against ischaemia/ reperfusion injury via the SAFE pathway. Hence, both HDL and S1P were given for 7 min during the 45 min reperfusion following 35 min of global ischaemia protected WT mice but failed to protect cardiomyocyte specific STAT-3 deficient and TNF α deficient mice. Furthermore, the infarct sparing effect with S1P was abolished in the presence of AG490, wortmannin but not PD98059. Interestingly, cytosolic levels of phosphorylated STAT-3 were decreased by all the inhibitors but phosphorylated levels of Akt and Erk were unchanged by S1P in the STAT-3^{-/-} mice. Our data demonstrate that cardioprotection induced by S1P may be conferred via a potential crosstalk between the SAFE and RISK protective paths.

4.1. HDL is cardioprotective against reperfusion injury via the SAFE pathway

HDL has been shown to be protective against ischemia reperfusion injury (Calabresi et al., 2003, Theilmeyer et al., 2006). HDL induced protection is associated with a reduction in cardiac TNF α levels and an increase of prostaglandins release (Calabresi et al., 2003). Prostaglandin release may contribute to the HDL induced reduction of TNF α or by exerting a vasodilatory action during reperfusion (Xiao et al., 2001, Calabresi et al., 2003). An *in vivo* mouse study demonstrated that HDL and S1P were protective against ischaemia/ reperfusion injury via the S1P₃ lysophospholipid receptor and nitric oxide-dependant pathway (Theilmeyer et al., 2006). These findings correlated with our study as STAT-3 is a known inhibitor of nitric oxide expression by interaction with NF κ B (Yu et al., 2002). Very recently, Frias et al. reported that native and reconstituted HDL induced STAT-3 activation in ventricular cardiomyocytes via the S1P₂ receptor and Erk1/2 phosphorylation (Frias et al., 2009). In this study we were interested whether HDL exerted its cardioprotective effects via the prosurvival SAFE pathway at reperfusion. Using a concentration of 200 μ g/ml, HDL protected the isolated perfused mouse heart. This beneficial effect was abolished in TNF^{-/-} and cardiac specific STAT-3^{-/-} mice, therefore suggesting that both TNF and STAT-3 are critical for protection with S1P. Furthermore, we observed an increase in mitochondrial STAT-3 levels following HDL

treatment at reperfusion, suggesting that HDL activation of mitochondrial STAT-3 contributes to the cardioprotective effect.

4.2. Cardioprotective role of S1P

A cardioprotective role for S1P at reperfusion was first suggested by Karliner et al. when S1P (0.4 μ M), given continuously during the 40 min of the reperfusion period (Vessey et al., 2008a), significantly reduced infarct size and showed an increase in phosphorylated levels of Akt. Our present data show that S1P, when given at a much lower final concentration (10 nM) for the first 7 min of reperfusion in a mouse model, was enough to reduce infarct size to a comparable degree observed in their rat model. Our choice of S1P concentration is also much lower than the physiologically levels of S1P which reside between 0.2 μ M and 1.1 μ M (Murata et al., 2000, Berdyshev et al., 2005), and thus limit the reported occurrence of coronary vasoconstriction, bradycardia and decreased blood pressure at higher S1P concentrations (Ohmori et al., 2003, Sanna et al., 2004, Karliner, 2004). We also observed this concomitant increase in phosphorylated Akt levels which affirms the involvement of this prosurvival kinase in S1P induced protection against reperfusion injury.

4.3. S1P protects against reperfusion injury by activation of the SAFE pathway

TNF is a pleiotrophic factor that exhibits both beneficial and deleterious effects. Previously, we have shown, that exogenous TNF can be cardioprotective against reperfusion injury in a time and concentration dependant manner (Lecour, 2009). The activation of TNF and STAT-3 which form the prosurvival SAFE pathway was successfully demonstrated in ischaemic postconditioning (Lacerda et al., 2009). The protective role of TNF was attributed to the TNF receptor 2, since receptor-specific deficient mice with TNF receptor 1 still intact, abolished the postconditioning effect. S1P failed to reduce the infarct size in TNF deficient mice indicating the involvement of this cytokine in mediating its protective effect. It is likely that S1P may be acting via the TNF receptor 2.

An increase in phosphorylated levels of STAT-3 has previously been suggested by the preconditioning effect mediated by S1P (Lecour et al., 2002). We show, for the

first time to our knowledge, a similar correlation between STAT-3 and S1P at reperfusion. Previous cardioprotective studies with pre- and postconditioning have delineated an essential role for STAT-3 to confer this beneficial effect (Lacerda et al., 2009, Lecour et al., 2005b). The presence of STAT-3 was originally identified only in the cytosol and nucleus but has been recently reported to also reside in the mitochondria (Wegrzyn et al., 2009). Although we detected phosphorylated STAT-3 in all these fractions, an increase in STAT-3 levels was only observed at 7 min in the mitochondria and disappeared by 15 min. This was in contrast to our findings in the cytosol and nucleus, therefore suggesting that the kinetic activation of STAT-3 in the mitochondria differs from the kinetic activation in the nucleus. In fact, our data cannot identify whether STAT-3 needs to be phosphorylated first in the cytosol before it translocates to the mitochondria or if STAT-3 can be phosphorylated directly in the mitochondria. Our findings are supported by a recent study from Boengler et al., whereby a cardioprotective role for mitochondrial STAT-3 against ischaemia/reperfusion injury was suggested, involving the stimulation of respiration and inhibition of the mPTP opening (Boengler et al., 2010).

4.4. S1P induced cardioprotection involves the activation of Akt and FOXO-1 but not Erk

The RISK pathway is well established in cardioprotection studies and mediated primarily by activation of the PI-3 kinase-Akt and MEK-Erk paths (Tsang et al., 2004, Hausenloy et al., 2005a). As discussed earlier, S1P induced an increase in phosphorylated Akt which was inhibited by wortmannin that also prevented infarct size reduction. Interestingly, we did not observe a similar outcome with Erk. Frias et al. demonstrated a stimulatory action of S1P on Erk in ventricular cardiomyocytes (Frias et al., 2009). In our experiments, inhibition of Erk with PD98059 did not abolish infarct size but reduced phosphorylated STAT-3 levels in the nuclear fraction. Our results may be explained by the possible STAT-3 active in the mitochondria that together with available phosphorylated Akt, were sufficient to significantly reduce cell death. FOXO-1 is a known downstream factor of Akt (Lekli et al., 2010). Similar to Akt, phosphorylated FOXO-1 levels were also increased with S1P. Furthermore, we show that inhibition of STAT-3 abolished this effect. This new insight suggests a dual

activation of FOXO-1 from both the SAFE and RISK paths. However, the individual contribution of either prosurvival pathway to its activation was not determined and is unknown.

4.5 Limitations of Study

STAT-3 can be phosphorylated at its serine 727 and tyrosine 705 residues. According to the current theory, tyrosine phosphorylation by JAK2 stimulation leads to STAT-3 dimerisation and translocation to the nucleus where it is additionally phosphorylated at its serine residue causing STAT-3 to leave the nucleus (Negoro et al., 2000, Mascareno et al., 2001, Kurdi and Booz, 2007). Recently however, STAT-3 has also been identified in the mitochondria phosphorylated at its serine residue. We examined the phosphorylated serine levels of STAT-3 in the cytosol, nucleus and mitochondria. It may be possible that the phosphorylated tyrosine levels of STAT-3 do not correlate with the serine levels under similar conditions.

Another limitation of this study is that we investigated the effect of S1P by abolishing STAT-3 in the nucleus and cytosol but not in the mitochondria. We have only shown an increase in phosphorylated STAT-3 levels with S1P in the mitochondria. Further studies could compare the mitochondrial respiration with S1P in the wildtype and STAT-3 deficient mice.

There are three S1P receptors in the heart, namely S1P₁, S1P₂, and S1P₃. These subtypes are differentially expressed across the various cells in the heart and several in vitro and in vivo studies provide evidence to support their cardioprotective role (Means and Brown, 2009). The inclusion of specific S1P receptor deficient mice and/ or inhibitors to our study would be useful to elucidate the upstream signalling mechanism leading to the activation of SAFE and RISK pathways.

4.6 Conclusion

High density lipoproteins (HDL) are known major plasma carriers of S1P (Argraves and Argraves, 2007). Sattler et al. have shown an association between S1P bound to HDL in healthy individuals which are decreased in coronary artery disease patients (Sattler et al., 2010). In addition, both HDL and S1P have been shown to protect against ischemia reperfusion (Theilmeyer et al., 2006) and the beneficial effects of HDL has been previously linked to the activation of STAT-3 (Frias et al.,

2009). Here, we show a protective signalling mechanism for S1P given at reperfusion that involves both the SAFE and RISK pathways (Figure 59). We can suggest that the beneficial effect of HDL may be attributed to the activation of this novel protective path. Clinical studies thus far, have been conducted with reconstituted HDL consisting of ApoA1 only. The addition of S1P to reconstituted HDL should also be considered for testing, especially in the context of ischaemic coronary artery disease. Future work will therefore aim at exploring this new protective path in HDL induced cardioprotection as this may present novel therapeutic opportunities against heart disease.

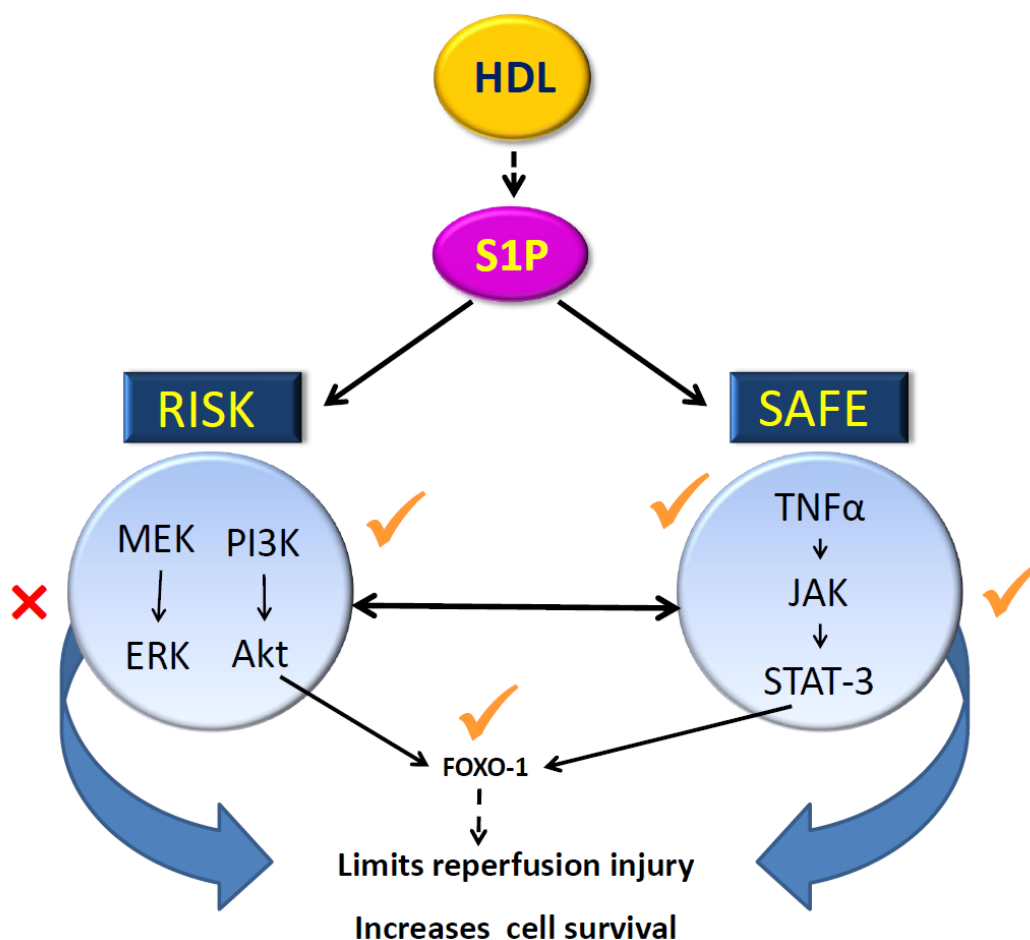


Fig 59. Summary of findings. HDL and S1P were both cardioprotective and the protective effect was mediated by a crosstalk between the SAFE and RISK pathways. *HDL*, High Density Lipoprotein; *S1P*, Sphingosine 1-Phosphate; *RISK*, Reperfusion Injury Salvage Kinase; *MEK*, Mitogen activated protein kinase Extracellular regulated Kinase; *ERK*, Extracellular Regulated Kinase; *PI3K*, Phosphoinositol 3-Kinase; *SAFE*, Survivor Activation Factor Enhancement; *TNFα*, Tumour Necrosis Factor alpha; *JAK*, Janus Kinase; *STAT-3*, Signal Transducer and Activator of Transcription-3.

G. FINAL DISCUSSION – CONCLUSION

1.0. Concluding remarks

Ischaemia reperfusion injury is a burden that affects a multitude of patients suffering from cardiovascular diseases. Experimentally, many strategies have been applied to reduce its harmful effects although they failed to translate to clinical practice. Ischaemic preconditioning (brief episodes of ischaemia and reperfusion prior to the prolonged ischaemic event) has limited potential as the ischaemic event cannot be premeditated. However, ischaemic postconditioning (brief episodes of ischaemia and reperfusion applied at the onset of reperfusion) is more clinically relevant and has been demonstrated to be nearly as powerful as preconditioning. The pitfall about ischaemic postconditioning is that the protocol has to be applied in a timely and precise manner following the ischaemic attack and adjustments may be required for the pathophysiology of the individual. This dilemma presents an opportunity to explore substitution of postconditioning induction in favour of a pharmacological agent that may be administered at the onset of reperfusion.

In this thesis, we were interested in delineating the protective signalling mechanisms involved in ischaemic and pharmacological postconditioning. Signal transducer and activator of transcription-3 (STAT-3) has been demonstrated as an important prosurvival factor in cardioprotection and we aimed to explore its role in postconditioning.

The main and novel findings are as follows:

1. Ischaemic postconditioning conferred cardioprotection in a murine model of reperfusion injury and conditions such as a minor difference in age, breeding strategy and number of postconditioning cycles were critical parameters to consider that may influence the outcome of postconditioning.
2. STAT-3 was essential for ischaemic postconditioning and mitochondrial translocation of the transcription factor was confirmed.
3. Pharmacological postconditioning with bradykinin, adenosine and opioids induced protection that was comparable to ischaemic postconditioning. Bradykinin and opioids required the activation of the SAFE pathway (TNF α and STAT-3) to mediate the protective effective effect. TNF α was not

essential to adenosine induced cardioprotection but STAT-3 was a prerequisite.

4. High Density Lipoprotein (HDL) and sphingosine-1-phosphate (S1P) are naturally occurring compounds that conferred cardioprotection when given at the onset of reperfusion via activation of the SAFE pathway. S1P induced protection involved the activation of both RISK (Akt) and SAFE pathways.
5. Our data confirmed the existence of possible crosstalk between multiple prosurvival signalling pathways that may work in parallel to exert the beneficial effects afforded by postconditioning.

In summary, we demonstrate the significance of STAT-3 in ischaemic and pharmacological postconditioning and also implicate activation of the SAFE pathway in cardioprotection (Figure 60).

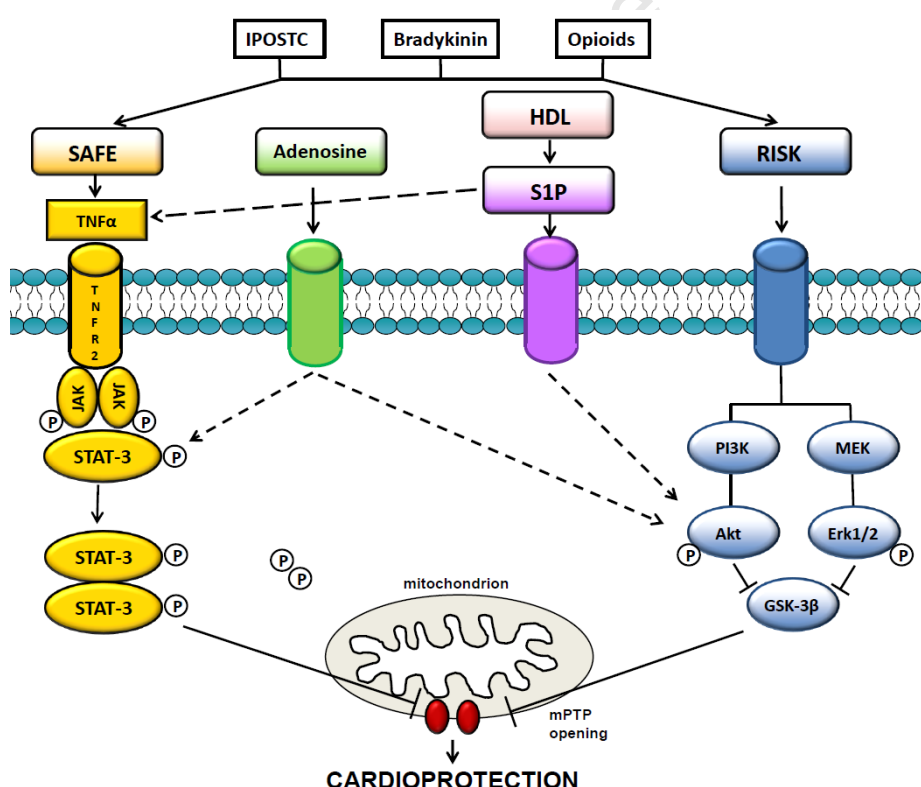


Fig 60. Schematic of proposed cardioprotective signalling mechanisms involving the SAFE and RISK pathways in ischaemic and pharmacological postconditioning. SAFE, Survival Activation Factor Enhancement; RISK, Reperfusion Injury Salvage Kinase; mPTP, mitochondrial permeability transition pore; TNF α , Tumour Necrosis Factor- α ; STAT-3, Signal Transducer and Activator of Transcription-3.

2.0. Limitations of Study

Our studies in this thesis employ the use of the Langendorff retrograde perfusion system which is an isolated heart model. As such, we are not aware of the complications that may arise in other organs and in the circulatory system, such as toxicity, reactive oxygen species formation and leukocyte infiltration. The effects on the vagal and sympathetic systems are also not considered. We would need to validate our experiments *in vivo*.

The signalling response of the RISK and SAFE pathways was blocked with use of non-specific inhibitors such as wortmannin and AG490. Wortmannin inhibits the PI3-kinase signalling path and does not act directly on Akt. AG490 is a JAK2 inhibitor which is located upstream of STAT-3. Since JAK2 may activate other signalling molecules in addition to STAT-3, blockade of this kinase is non-specific and does permit identification of downstream targets.

Phosphorylation of STAT-3, Akt, ERK and FOXO-1 were investigated in the nucleus, cytosol and/ or the mitochondria. It is important to note that activation of these signalling factors in these cellular compartments may provide different effects that may also occur at different times. This represents a challenge to elucidate the correct and appropriate activation that may be required for specific downstream responses in the treatment and management of cardiovascular disease.

3.0. Future Directions

Many pharmacological agents demonstrated to mimic the protective effects of ischaemic preconditioning have also been shown to offer the similar benefits as postconditioning agents. It is likely that resveratrol and ethanolamine, that I have successfully shown as preconditioning agents would also translate to postconditioning agents treated at reperfusion.

We have presented native high density lipoprotein (HDL) and sphingosine-1-phosphate (S1P) as novel cardioprotective agents. Since S1P forms a protective component of HDL, it would be interesting to test ability of reconstituted HDL that contained a higher concentration of S1P for a desired optimal protection levels against reperfusion injury. In addition, a chronic study involving the administration of

this novel molecule would allow us to explore its potential long term benefits. This study may also be conducted in STAT-3 deficient mice to determine if over a sustained period, a parallel pathway may develop to compensate for the observed loss of acute cardioprotection.

S1P has 3 main receptors in the heart, S1P₁₋₃. We have shown that S1P protects via a crosstalk between the SAFE and RISK pathways and the use of specific agonists and antagonists for these receptors could provide useful information on the signalling cascade involved that may aid the development of novel therapeutics.

There is a high prevalence of heart attacks occurring among obese and diabetic patients. It would be enlightening to investigate the ability of HDL and S1P to elevate depleted levels of HDL in a murine model of obesity and diabetes and its potential protective effect on the heart against ischaemia reperfusion injury.

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H. PUBLICATIONS ARISING FROM THIS WORK

Age, genetic characteristics and number of cycles are critical factors to consider for successful protection of the murine heart with postconditioning. **Somers S**, Lacerda L, Opie L, Lecour S. *Physiol Res*. 2011 Oct 12. (in press)

Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol- and melatonin-induced cardioprotection. Lamont KT, **Somers S**, Lacerda L, Opie LH, Lecour S. *J Pineal Res*. 2011 Feb 23. 2011 May;50(4):374-80.

Ethanolamine is a novel STAT-3 dependent cardioprotective agent. Kelly RF, Lamont KT, **Somers S**, Hacking D, Lacerda L, Thomas P, Opie LH, Lecour S. *Basic Res Cardiol*. 2010 Nov;105(6):763-70.

Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. Lacerda L, **Somers S**, Opie LH, Lecour S. *Cardiovasc Res*. 2009 Nov 1;84(2):201-8.

I. PREVIOUS PUBLICATIONS

TNF α -induced cardioprotection is independent of the activation of the prosurvival kinase Erk. **Somers S**, Engerer A, Lecour S. *SA Heart* 2009 6: 168-172.

The PGE2-Stat3 interaction in doxorubicin-induced myocardial apoptosis. Frias MA, **Somers S**, Gerber-Wicht C, Opie LH, Lecour S, Lang U. *Cardiovasc Res*. 2008 Oct 1;80(1):69-77.

Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. Suleman N, **Somers S**, Smith R, Opie LH, Lecour SC. *Cardiovasc Res*. 2008 Jul 1;79(1):127-33.

Pharmacological preconditioning with tumor necrosis factor- α activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). Lecour S, Suleman N, Deuchar GA, **Somers S**, Lacerda L, Huisamen B, Opie LH. *Circulation*. 2005 Dec 20;112(25):3911-8.

J. PEER REVIEWED, PUBLISHED CONFERENCE PROCEEDINGS

- 2007: "Critical role of age, strain and number of cycles in ischaemic postconditioning". **Sarin Somers**, Lydia Lacerda, Naushaad Suleman, Lionel Opie, Sandrine Lecour. International Society for Heart Research; Italy; *published in J Mol Cell Cardiol, 2007; Vol 42, S186*
- 2008: "Myocardial preconditioning with Sphingosine-1-Phosphate, a major component of HDL, protects against ischemia via STAT-3 activation". Jonathan King, **Sarin Somers**, Damian Hacking, Roisin Kelly and Sandrine Lecour. 9th Annual Congress of SA Heart Association, South Africa; *published in SA Heart, Spring 2008; Vol 5, No. 4; p216*
- 2008: "Sphingosine-1-phosphate (S1P) can mimic ischemic postconditioning via activation of the JAK/STAT-3 pathway". **Sarin Somers**, Lionel Opie and Sandrine Lecour. 9th Annual Congress of SA Heart Association, South Africa; *published in SA Heart, Spring 2008; Vol 5, No. 4; p244*
- 2009: "Pharmacological postconditioning with TNF alpha protects via the SAFE pathway rather than the RISK". Lydia Lacerda, **Sarin Somers**, Lionel H Opie, Sandrine Lecour. European Society of Cardiology - Heart Failure Conference – ISHR section; France; *published in European J Heart Failure, 2010; Vol 12(1).*
- 2009: "Innate immunity and protection against reperfusion injury via the SAFE pathway". Lydia Lacerda, **Sarin Somers**, Lionel Opie, Sandrine Lecour; 10th Annual Congress of SA Heart Association, South Africa; *published in SA Heart, Spring 2009; Vol 6, No. 4; p272*
- 2009: "Signaling pathways activated in HDL-mediated cardioprotection: is it SAFE or at RISK?". **Sarin Somers**, Lionel Opie and Sandrine Lecour; 10th Annual Congress of SA Heart Association, South Africa; *published in SA Heart, Spring 2009; Vol 6, No. 4; p293*
- 2010: "The SAFETY and RISKS of sphingosine-1-phosphate induced cardioprotection". **Sarin Somers**, Lionel Opie, Sandrine Lecour. International

Society for Heart Research; Japan; *published in J Mol Cell Cardiol*, 2010; Vol 48, S39

2010: “Bradykinin, insulin and opioids mimic ischaemic postconditioning via the SAFE pathway. Lydia Lacerda, **Sarin Somers**, Lionel Opie, Sandrine Lecour. Frontiers in Cardiovascular Biology, Berlin, Germany, 2010 – *published in Cardiovasc Res*, Vol 87; Supplement 1, 15th July 2010

2010: “HDL protects against lethal reperfusion injury via the SAFE pathway”. Miguel Frias, **Sarin Somers**, Lydia Lacerda, Richard James and Sandrine Lecour. 11th Annual Congress of SA Heart Association, South Africa; *published in SA Heart*, Winter 2010; Vol 7, No.3; p202

K. CONFERENCE OUTPUTS

Ischaemic postconditioning requires STAT-3 for cardioprotection. **Sarin Somers**, Naushaad Suleman, Lionel Opie, Sandrine Lecour. Physiological Society of Southern Africa, Durban, South Africa; 2006

Critical role of age, strain and number of cycles in ischaemic postconditioning. **Sarin Somers**, Lydia Lacerda, Naushaad Suleman, Lionel Opie, Sandrine Lecour. International Society for Heart Research; Italy; 2007

Critical role of age, strain and number of cycles in ischaemic postconditioning. **Sarin Somers**, Lydia Lacerda, Naushaad Suleman, Lionel Opie, Sandrine Lecour. Physiological Society of Southern Africa, Gauteng, South Africa; 2007

Postconditioning requires STAT-3 to confer cardioprotection. **Sarin Somers**, Lionel Opie, Sandrine Lecour. South African Medical Research Council Research Day, Cape Town, South Africa; 2008

Myocardial preconditioning with Sphingosine-1-Phosphate, a major component of HDL, protects against ischemia via STAT-3 activation. Jonathan King, **Sarin Somers**, Damian Hacking, Roisin Kelly and Sandrine Lecour. 9th Annual Congress of SA Heart Association, South Africa; 2008

Sphingosine-1-phosphate (S1P) can mimic ischemic postconditioning via activation of the JAK/STAT-3 pathway. **Sarin Somers**, Lionel Opie and Sandrine Lecour. 9th Annual Congress of SA Heart Association, South Africa; 2008

Pharmacological postconditioning with TNF alpha protects via the SAFE pathway rather than the RISK. Lydia Lacerda, **Sarin Somers**, Lionel H Opie, Sandrine Lecour. European Society of Cardiology - Heart Failure Conference – ISHR section; France; 2009

The SAFETY and RISKS of sphingosine-1-phosphate induced cardioprotection. **Sarin Somers**, Lionel Opie, Sandrine Lecour. World Congress of Cardiology; China; 2009

Innate immunity and protection against reperfusion injury via the SAFE pathway. Lydia Lacerda, **Sarin Somers**, Lionel Opie, Sandrine Lecour; 10th Annual Congress of SA Heart Association, South Africa; 2009

Signaling pathways activated in HDL-mediated cardioprotection: is it SAFE or at RISK? **Sarin Somers**, Lionel Opie and Sandrine Lecour; 10th Annual Congress of SA Heart Association, South Africa; 2009

Sphingosine-1-phosphate (S1P) can mimic ischaemic postconditioning via activation of the STAT-3 pathway. **Sarin Somers**, Lionel Opie and Sandrine Lecour; European Society of Cardiology, Barcelona, 2009

The SAFETY and RISKS of sphingosine-1-phosphate induced cardioprotection". **Sarin Somers**, Lionel Opie, Sandrine Lecour. Physiological Society of Southern Africa, Stellenbosch, South Africa; 2009

Signaling pathways activated in HDL-mediated cardioprotection: is it SAFE or at RISK? **Sarin Somers**, Lionel Opie and Sandrine Lecour; University of Cape Town/ Groote Schuur Medical Research Day, South Africa; 2009

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